In vitro modelling of neurodegeneration and neuroprotection specific for Alzheimer's disease in cell cultures

Ph. D. Thesis

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2005

Abbreviations:

Aβ = beta amyloid peptide

AC = Aβ cluster

AD = Alzheimer's disease

apoE4 = apolipoprotein E4

APP = amyloid precursor protein

ASCOM = amyloid surface covering molecules

BACE = beta-amyloid cleavage enzyme (β-secretase)

BSA = bovine serum albumin

ConA = concanavalin-A

CR = Congo red

DHE = Dihydro ethidine

DMEM = Dulbecco`s modified Eagle`s medium

DMSO = dimethyl-sulfoxide

H_2O_2 = hydrogen-peroxid

LPYFDn = LPYFD-amide

MAP2 = microtubule-associated protein 2

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N = neurite

NAC = non-Aß component of AD amyloid

OD = optical density

P = perikaryon

PBS = phosphate buffered saline

pGn = GGGGG-amide

PIC2 = "Phosphatase Inhibitor Cocktail 2"

PS–γ = presenilin–γ-secretase complex

RA = all-trans-retinoic acid

RP = rounding perikaryon

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Datki Z, Juhasz A, Galfi M, Soos K, Papp R, Zadori D, Penke B. 2003. *Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells.* Brain Res Bull. 62, 223-9.

Fulop L, Zarandi M, **Datki Z**, Soos K, Penke B. 2004. *Beta-amyloid-derived pentapeptide RIIGLa inhibits Abeta(1-42) aggregation and toxicity.* Biochem Biophys Res Commun. 324, 64-9.

Datki Z, Papp R, Zadori D, Soos K, Fulop L, Juhasz A, Laskay G, Hetenyi C, Mihalik E, Zarandi M, Penke B. 2004. *In vitro model of neurotoxicity of Abeta 1-42 and neuroprotection by a pentapeptide: irreversible events during the first hour.* Neurobiol Dis. 17, 507-15.

Kellermayer MS, Grama L, Karsai A, Nagy A, Kahn A, **Datki ZL**, Penke B. 2005. *Reversible mechanical unzipping of amyloid beta -fibrils.* J Biol Chem. 280, 8464-8470.

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Zsolt Datki, Katalin Soós, Anna Juhász, Zsolt Molnár, Botond Penke, 2002. *The neurotoxic mechanism of β-amiloid peptides (Theorethical research).* 6th Hungarian Conference on Alzheimer's Disease, Clinical Neuroscience, 55. 1, 13-14.; Győr, Hungary.

Zsolt Datki, Katalin Soós, Anna Juhász, Tibor Harkányi, Gábor Laskay, Botond Penke. *Pitfalls of neurotoxicity investigation of β-amyloid 1-42 peptide in vitro and in vivo.* 27th European Peptide Symposium (Sorrento, Italy; 2002), Journal Peptide Science. 8, S 170/P C98.

Zsolt Datki, Rita Papp, Katalin Soós, Dénes Zádori, Anna Juhász, Gábor Laskay, Csaba Hetényi, Erzsébet Mihalik, Botond Penke. 2003. *In vitro model of neurotoxicity of Aβ 1-42 and neuroprotection by a small peptide: irreversible events during the first hour.* 7 th Jubille Hungarian Conference on Alzheimer's Disease, Clinical Neuroscience, 56. 9-10, 332.; Budapest, Hungary.

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B. Penke, **Z. Datki,** C. Hetényi, Z. Molnár, I. Lengyel, K. Soós and M. Zarándi, 2003. *Molecular pathomechanisms of Alzheimer's disease*, Journal of Molecular Structure: THEOCHEM, 29, 507-513.

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Katalin Soós, Botond Penke, **Zsolt Datki,** Livia Fülöp, Márta Zarándi. *An improved synthesis of Beta-amyloid peptides for in vitro and in vivo experiments.* 28 th European Peptide Symposium (Prague, Czech Republic; 2004), Journal Peptide Science. 10, 136/P61.

1. Introduction

1.1. Characterization of the Alzheimer's disease

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and prion diseases are increasingly realized to have common cellular and molecular mechanisms; can be characterized by aggregating proteins (Ross *at al.*, 2004), inclusion body formation (Bossy-Wetzel *et al.*, 2004), axon (*in vivo*) and neurite (*in vitro*) degeneration (Sheetz *et al.*, 1998; Roy *et al.*, 2005), and apoptosis (Stoppini *et al.*, 2004).

AD is mostly a late-onset dementing illness, with progressive loss of memory, task performance, speech, and recognition of people and objects (Davison A.N. 1987; Ross *et al.*, 2004). In AD, neurons of the hippocampus and cerebral cortex are selectively lost. Brains of individuals with AD manifest two characteristic lesions: extracellular amyloid (or senile) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Goedert *et al.*, 1989; Selkoe, D.J., 2003). Amyloid plaques contain small, toxic cleavage peptides of the amyloid precursor protein (APP; 770 amino acids). These peptides consist of 40-42 amino acids and called β-amyloid peptides (Aβ). The alpha-synuclein (major component of Lewy bodies in Parkinson's disease) is also present in Alzheimer's amyloid plaques. The toxic cleavage product of the alpha-synuclein is the non-Aß component of AD amyloid (NAC), which had previously been identified as a component of AD amyloid plaques. (Kim et al., 2004; Lavedan, C. 1998; Iwai A. 2000) The apolipoprotein E4 (apoE4) genotype is a powerful risk factor for developing AD, and it may possibly affect Aβ deposition and neurofibrillary tangle formation (Roses, A.D., 1996).

APP is a type I. membrane protein and contains a large extracellular region, a transmembrane helix and a short cytoplasmic tail. The N-terminal half of APP contains a heparin-binding domain, a copper-binding domain and an APP protease inhibitor domain. (Rossjohn, *et al.*, 1999). Toxic Aβ originates from regulated intramembrane proteolysis of APP by a complex of secretases (Fig. 1.). The first cleavage of APP is mediated by β- or α-secretase, releasing most of the extracellular portion of APP as two fragments, APPs-α and APPs-β, leaving behind the C-terminal membrane bound fragment. This portion of APP is then cleaved by a large protein complex, γ-secretase, at several sites including amino acid at 711 (Aβ 1-40) and at 713 (Aβ 1-42). The amino acid sequence of the Aβ 1-42:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

In AD, the oxidative stress, the mitochondrial dysfunction and as a result the energy deficit may contribute to impaired clearance of protein aggregates and neuronal dysfunction, affecting ion channel and pump activity, neurotransmission, and axonal and dendritic transport. (Bossy-Wetzel *et al.*, 2004)

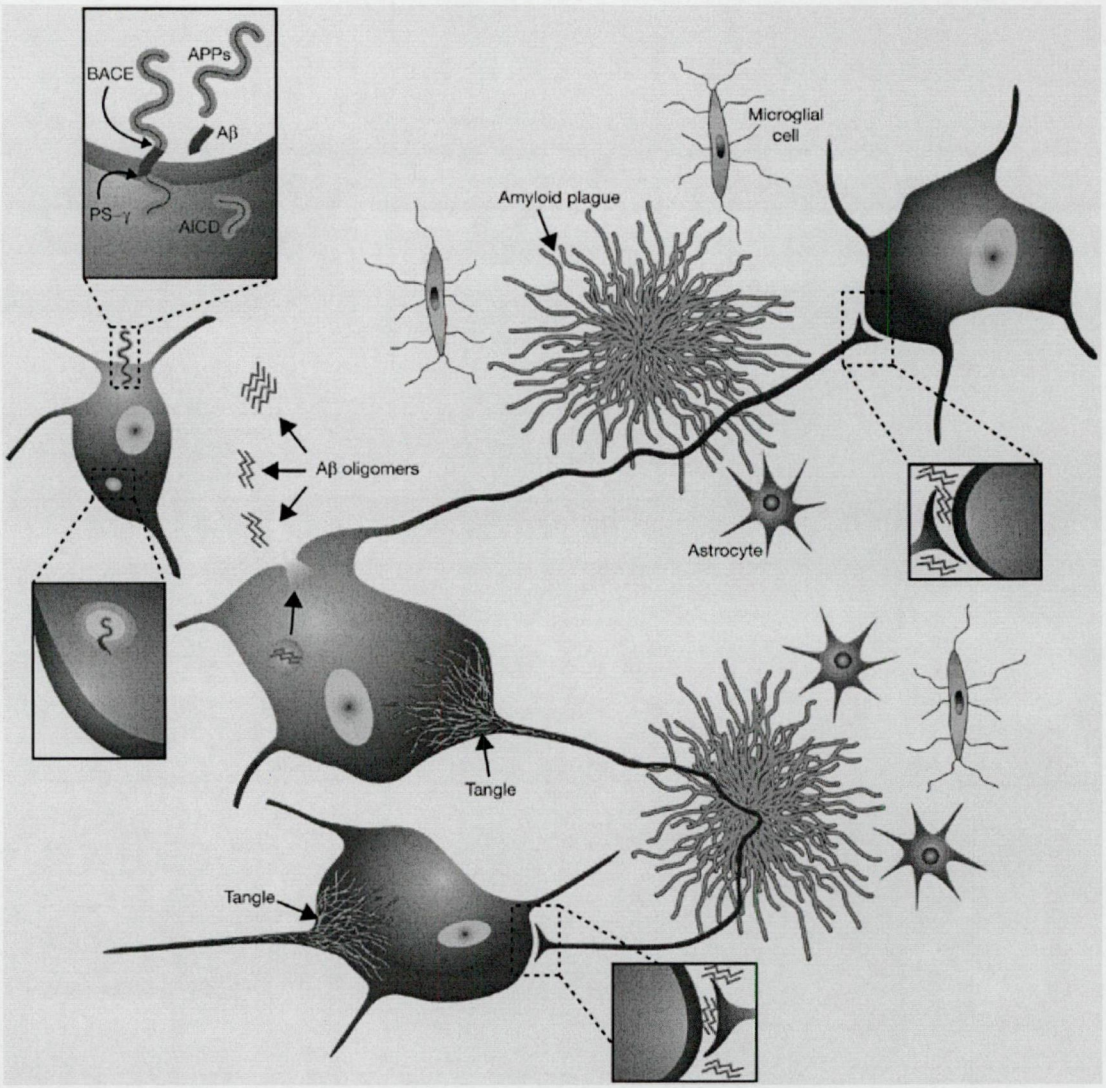


Fig. 1. *A model of key events underlying the pathogenesis of AD, based on available evidence. APP molecules on the plasma membrane and in intracellular vesicles, such as endosomes, are cleaved by β-secretase (BACE) and the presenilin−γ-secretase complex (PS−γ) to liberate the Aβ. A portion of Aβ peptides can oligomerize, initially intravesicularly, and can be released into the interstitial fluid of the brain, where soluble oligomers may diffuse into synaptic clefts and interfere with synaptic function by unknown mechanisms. Aβ oligomers can further polymerize into insoluble amyloid fibrils that aggregate into spherical plaques, resulting in tortuosity and dysfunction of adjacent axons and dendrites. A major accompaniment of such events is the activation of kinases in the neuronal cytoplasm, leading to the hyperphosphorylation of the microtubule-associated protein, tau, and its polymerization into insoluble filaments (as neurofibrillary tangles). (Dennis J. Selkoe, 2004)*

1.2. Relations of the in vitro cell breeding techniques and the Alzheimer's disease

β-Amyloid peptide (Aβ) aggregates are toxic on neurons (Malouf *et al.*, 1992). Aβ fibrils also interact with microglial cells and monocytes and stimulate expression of proinflammatory genes (Combs *et al.*, 2001). There is a need for an easy and reliable method to measure the toxic effects of Aβ peptides and similar toxic polypeptide aggregates (e.g. NAC, prion peptides, human amylin, etc.). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been widely used to measure the toxicity of different substances in cell cultures (Schiff *et al.*, 1985). MTT enters the cells by endocytosis and is reduced to formazan by NADH reductase and other enzymes, which can be measured spectrophotometrically. All subcellular fractions can reduce MTT when supplied with NADH or NADPH. The amount of formazan reflects the reductive potential of the cytoplasm and the cell viability. (Liu *et al.*, 1997a) This method has proven to be very useful in neurotoxicity studies of Aβ peptides (Liu *et al.*, 1997b). In most cases, neuroblastoma cells, e.g. SH-SY5Y, are applied (Cedazo-Minguez *et al.*, 2001). The MTT assay is rapid and generally shows a good correlation with other viability tests (Loske *et al.*, 1998; Soriano *et al.*, 2003) and *in vivo* results (Ghiso *et al.*, 2002; Abe *et al.*, 1998).

The neurotoxicity of Aβ-peptides (Aβ 1-42 and Aβ 25-35) has been determined with the MTT assay on PC-12 (Wei *et al.*, 2000), C 1300 (Takenouchi *et al.*, 1998) and SH-SY5Y (Webster *et al.*, 2002) cells. Mostly non-differentiated neuroblastoma cells have been used in the MTT assay. Aβ and other aggregated peptides (NAC, prions and human amylin) exert toxic effect by inducing apoptosis in neuroblastoma cells (Li *et al.*, 1996; Chabry *et al.*, 2003; O'Donovan *et al.*, 2001; Rumora *et al.*, 2002).

Working with non-differentiated cells involves some disadvantages. The number of cells is continuously growing during the experiments (e.g. Aβ-treatment for 24 h or 48 h), so the results of MTT assay ought to be corrected. Yankner *et al.* reported in 1990 that Aβ-peptides were neurotrophic to undifferentiated neurons and neurotoxic to mature ones. In cultures, the cells are unsynchronized, which means uncertainty in toxicity measurements. The MTT assay of Aβ-peptides might lead to false results when non-differentiated neuroblastoma cells are used, because Aβ-peptides act mostly on the neurites of the neuroblastoma cells (Kumar *et al.*, 2000; Nordin-Andersson *et al.*, 1998). We had the same findings in our experiments.

Cell membrane proteins (receptors) play key role in the events that lead to apoptotic cell death after Aβ treatment. Cell surface receptor complexes are mediating the cellular effects of Aβ peptides (Bamberger *et al.*, 2003); the membrane composition of differentiated and non-differentiated cells are different. As a consequence, we have now attempted to improve the weak points of the MTT method by using differentiated cells. SH-SY5Y cells were incubated with all-*trans*-retinoic acid (RA); the resulting differentiated cell culture was used for neurotoxicity studies (Lambert et al., 1994). Our aim was to find a reliable *in vitro* assay for neurotoxicity measurements, an MTT assay characterized with two improvements: 1) use of highly differentiated neuroblastoma cells which are (morphologically and physiologically) very resemble to neurons. 2) Use of constant cell number during the whole experiment; in this case the redox activity of the cells is directly proportional to the neurotoxicity of the substances and no correction of the results is needed. We wanted to find a method generally suitable for measurements of neurotoxicity of very different aggregating peptides like β-amyloids or NAC.

AD is a synaptic failure caused by the diffusible oligomeric assemblies of the 40-43 aminoacid-long β-amyloid (Aβ; Selkoe, 2002). Aβ is neurotoxic (Lambert *et al.*, 1994; Wei *et al.*, 2000), induces axonopathy (Higuchi *et al.* 2002; Terwel *et al.*, 2002) and neuronal apoptosis (Loo *et al.*, 1993; Morishima *et al.*, 2001) both in brain tissue (Vickers *et al.*, 2000) and in neuroblastoma cells *in vitro* (Li *et al.*, 1996; Mookherjee *et al.*, 2001). Reliable *in vitro* assays are essential in the investigation of the effects of neurotoxic compounds such as Aβ. Axonopathy is an early marker of Aβ neurotoxicity, in contrast to apoptosis. The measurement of neurite degeneration of differentiated SH-SY5Y cells is a useful model for the *in vitro* identification of axonopathy-inducing substances (Nordin-Andersson *et al.*, 1998).

The axonal disruption induced by Aβ is strongly related to the hyperphosphorylation and dissociation of the microtubule-associated tau protein (Tomidokoro *et al.*, 2001). The phosphorylated tau protein in human cerebrospinal fluid is a diagnostic marker for AD (Ishiguro *et al.*, 1999). Tau protein is hyperphosphorylated in human-derived SH-SY5Y cells during apoptosis (Mookherjee *et al.*, 2001). This process is essential for Aβ-induced neurotoxicity (Rapoport *et al.*, 2002): Aβ amyloidosis evokes the initial stage of tau accumulation (Tomidokoro *et al.*, 2001). However, no data has been published so far concerning the initial events of the cellular neurotoxicity of Aβ, such as the time course of the events, the changes in tau phosphorylation and cell viability, and the reversible or irreversible character of these changes.

In this study, we report the early events of neurite degeneration and intracellular changes induced by the 42 aminoacid long peptide (Aβ 1-42) in the differentiated SH-SY5Y neuroblastoma cell line and the neuroprotective effect of a new pentapeptide, LPYFD-amide (LPYFDn). The pentapeptide LPYFDn was designed on the basis of Soto's well-known BSB-peptide LPFFD (Soto *et al.*, 1998), replacing one of the phenylalanines to tyrosine and the C-terminal $-COO^-$ anion to $-CONH_2$ group. These changes presumably increase the binding affinity of the pentapeptide to Aβ aggregates. Various methods (MTT and Neutral Red bioassay, light and fluorescence microscopy, morphological studies, scanning electron microscopy, immunocytochemistry and the Congo red (CR) aggregation assay) were used to study the neurotoxic events involving Aβ 1-42.

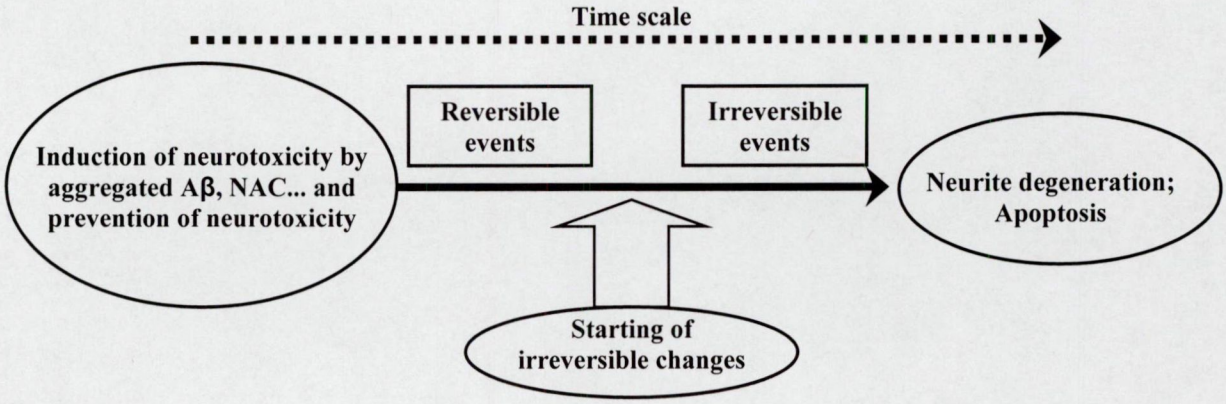
2. Aims

Our aim was the *in vitro* study of the neurodegeneration process, caused by aggregated polypeptides and proteins specific for Alzheimer's disease. The following experiments were used for modelling the disease in cell cultures:

1. Use of human neuroblastoma cells has proven to be a good model for neurotoxicity studies. However, non-differentiated blastoma cells are not identical with neurons containing much less membrane receptors. Our aim was to work out a neurotoxicity test using fully differentiated SH-SY5Y human derived neuroblastoma cells;
2. The exact mechanism of the neurotoxicity caused by aggregated peptides (Aβ, NAC) is not yet known. Our second aim was searching and modelling of the neurotoxic events after treatment of the cells with different aggregating peptides in *in vitro* cell culture;
3. During the neurotoxicity process there might be first reversible and after that irreversible events. Our third aim was the identification of the irreversibility point (in time and cell physiology after treatment with Aβ 1-42), which is the critical moment in neurodegeneration, because after these event the cell death (apoptosis) is inescapable;
4. There are already several short Aβ-fragments and fragment analogs possessing neuroprotective activity against Aβ peptides. In our laboratory a series of peptides were designed and synthesized. Their protective effects were measured and the best pentapeptide (LPYFDn) was intensively studied using different methods.

   On the base of the results of the above summarised experiments a hypothesis for the *in vitro* neurotoxicity of aggregated Aβ 1-42 was created.

We have studied the following hypothetic mechanism of neurodegeneration caused by aggregated peptides and proteins:



3. Materials and methods

3.1. Materials

Aβ peptides (1-42, 1-40, 4-42, 5-42, 42-1, 25-35, 31-35, all-D 1-40, MIX 1-42), NAC (non-A beta component), LPYFD-amide (LPYFDn), GGGGG-amide (pGn), GRGDS-amide (GRGDSn), FRHDS-amide (FRHDSn), KLVFF-amide (KLVFFn) and RIIGL-amide (RIIGLn) were synthesized in our laboratory at the Department of Medical Chemistry, University of Szeged, Hungary. Solvents were purchased from Merck Europe. Milli-Q_{PLUS} distilled water was used in each experiment. Congo red (CR), Triton 100X, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Neutral Red, all-*trans*-retinoic acid (RA), concanavalin-A (ConA), bovine serum albumin (BSA), Pluronic acid, Fura-2 AM, Dihydro ethidine (DHE), C11 BODIPY$^{581/591}$, ethidium bromide, polyclonal antibody pSer^{396} Anti-Phospho-Tau developed in rabbit, mouse monoclonal Anti-Human Amyloid Beta Protein, monoclonal Anti-MAP2 (2a+2b) antibody produced in mouse, FITC Conjugate-Goat Anti-Rabbit IgG, FITC Conjugate-Anti-Mouse IgG and "Phosphatase Inhibitor Cocktail 2" (PIC2) were obtained from Sigma-Aldrich (Budapest).

3.2. Experimental treatments of differentiated SH-SY5Y cells

The non-differentiated SH-SY5Y cell line was bought from Sigma-Aldrich (Budapest). The cells were grown to confluency at 37 °C for 8 days in Dulbecco's modified Eagle's medium (MEM):F-12 (1:1) containing phenol red (Sigma-Aldrich; Budapest) on 24- (or 96-) well plates (Nunc; Roskilde, Denmark) in a humidified atmosphere containing 5% CO_2. L-Glutamine (4 mM; Gibco; Budapest), penicillin (200 units/ml; Gibco; Budapest), streptomycin (200 μg/ml; Gibco; Budapest), MEM non-essential amino acids (Gibco; Budapest), and 10 μM all-*trans* retinoic acid (RA; Sigma-Aldrich; Budapest) were dissolved in DMSO. The final concentrations of all-*trans* retinoic acid and DMSO were 10 μM and 0.5%, respectively, and 10% FBS (Gibco; Budapest) that were added to the medium. On the first day, the number of non-differentiated cells in the wells were 2.5 x 10^5 cells/ml. After 8 days of differentiation, the cells attached to the plate as a monolayer, and cell counting indicated 3 x 10^5 cells/cm^2 (corresponding to 6.5 x 10^5 cells/ml in suspension). Before treatment, each peptide was ultrasonicated in the culture medium (2% FBS) for 10 min. This medium was free from phenol red and differentiating agents (RA, DMSO). The aggregation of Aβ peptides were performed in

aqueous solution (10^{-5} M) by gentle shaking at room temperature for 24 hours; after aggregation the colloidal solution was lyophilized (Fig. 9 shows the fibrillar structure of aggregated Aβ peptides). Differentiated neuroblastoma cells (Datki *et al.*, 2003) were incubated with the following peptides: **a.** 10 μM aggregated Aβ peptides; **b.** 10 μM aggregated NAC ; **c.** a mixture of 10 μM aggregated Aβ 1-42 and 50 μM LPYFDn; **d.** 10 μM aggregated Aβ 1-42 and 50 μM pGn in a 24- (or 96-) well plate for 24, 48 or 72 hours. The original supernatant solution was removed from the cells with a pipette and the new medium (1 ml, 2% FBS, 37 °C, containing aggregated Aβ peptides on their own or with the pentapeptides) was added immediately (within 3 s) to the wells at 37 °C. Sedimented Aβ clusters remain cell-bound after washing of the cells (Fig. 9/A2). (Paraformaldehyde-fixed cells can not bind Aβ clusters.)

3.3. MTT assay using non-differentiated SH-SY5Y neuroblastoma cells

A slightly modified method of Loske *et al* (1998) was used in our first experiments. The SH-SY5Y cells used here were obtained from Sigma-Aldrich (Budapest). Cells were plated for 24 h at 37 °C on 96-well plates (Nunc, Roskilde, Denmark) at a density of 3 x 10^4 cells/well, to confluency, with 5% CO_2 in a humidified atmosphere with culture medium. Figure 3/A and 4. show the short neurites of the non-differentiated cells. 10 μl of aqueous MTT solution (4 mg/ml) was then added to each well (100 μl), and the mixture was incubated at 37 °C for 3 hours. The MTT solution was carefully decanted off, and formazan was extracted from the cells with 100 μl of a 4:1 DMSO-EtOH mixture in each well. Colour was measured with a 96-well ELISA plate reader at 550 nm, with the reference filter set to 620 nm. All MTT assays were repeated three times.

3.4 Improved MTT assay, using differentiated SH-SY5Y neuroblastoma cells

The same cell culture was used with the following changes: cells were grown for 8-10 days on 24-(or 96-) well plates, and cell differentiation was initiated by the addition of 10 μM RA dissolved in cell culture medium containing 0.5% DMSO. On the first day, the number of non-differentiated cells in the wells was 2.5 x 10^5 cells/ml in suspension. After 8-10 days of differentiation, the cells were attached as a monolayer to the plate, and cell counting gave a number of 3 x 10^5 cells/cm^2 (corresponding to 6.5 x 10^5 cells/ml in suspension). Figure 3B shows the confluent monolayer of the cells with long neurites (3/C and 4.) and branching

neurite terminates (3/D). The supernatant solution was removed with a pipette and a new medium (with aggregating peptides, pentapeptides), free from phenol red and differentiating agents such as RA and DMSO, was added immediately to each well (within 3 s) at 37 °C. 100 µl of MTT stock solution (4 mg/ml) was added in each well, containing about 1 ml cell culture medium, and the mixture was incubated for 3 hours. The MTT solution was carefully decanted off and formazan was extracted from the cells with 1 ml of a DMSO/EtOH (4:1) mixture in each well. The color intensity of formazan was measured with a 96-well ELISA plate reader at 550 nm with the reference filter set to 620 nm. All MTT assays were performed in triplicate; one measurement contained 7 parallels (n=21).

3.5. [^{3}H]-Thymidine incorporation was measured by the method of Volm *et al.* (1979).

3.6. Cell adhesion assay

Cell adhesion assay was essentially performed as previously described by Luque *et al.* (1994). Differentiated neuroblastoma cells in suspension form were incubated with the following peptides: **a.** anti-integrin β1 antibody (1:100); **b.** 10 µM aggregated Aβ 1-42; **c.** 10 µM GRGDSn; **d.** 10 µM FRHDSn; e. 10 µM KLVFFn; 10 µM RIIGLn and 10 µM LPYFDn in Eppendorf tubes for 20 min (Fig. 27). Cells were plated for 2 h at 37 °C on 96-well plates at a density of 3 x 10^4 cells/well, to confluency, with 5% CO_2 in a humidified atmosphere with Dulbecco's modified Eagle's medium (MEM): F-12 (1:1). Nonbound cells were removed by rinsing the wells with serum-free medium, whereas bound cells were fixed with methanol, and were washed twice with PBS. The cells were stained with 10 µM Cresyl Violet (2 h) and were counted per unit area with the aid of an inverted light microscope, using a 20x high-power objective and a digital-camera grid (calibrated analysis 3.2 image-computer program). Experimental treatments were performed in triplicate with a minimum of three areas counted per well, seven wells per cases (n=63).

3.7. The protein content of the cells was measured with a Lowry assay as modified by Huemer *et al.* (1970).

3.8. Monolayer primary neurocyte cultures

Primary monolayer neurocyte cultures were prepared from rat brain cells, by the methods of Sensenbrenner *et al.* (1984), Durko *et al.* (1987) and Janka *et al.* (1980). The neurocyte cultures were maintained at 37 °C in humidified atmosphere of 5 % CO_2 in air. The culture medium was changed in every third day. Cell viability was 90 to 100 %, and the cell number was determined as 2 x 10^5 cells/ml.

3.9. Morphological evaluation

In vitro neurite degeneration induced by Aβ 1-42 was measured by the morphological analysis of the neurites and the cell bodies of differentiated cells (Fig. 12 - 14). The morphology of the neurites (qualitatively complete neurites), according to their thickness and straightness, and the morphology of the cell bodies, according to their shape and diameter, were observed. Each cell was monitored continuously and photographed every 30 s from 0 min to 60 min during the different treatments at 460x magnification. Altogether one hundred cells were analyzed. After the treatment, the differentiated cells were fixed with 1% paraformaldehyde for 20 min, and the nuclei were labeled with ethidium bromide (20 μg/ml) for 10 min in a 1:5 mixture of DMSO/EtOH. The number and localization of the labeled nuclei were matched the number and localization of the cell bodies analyzed morphologically. Measurements were performed with a light-fluorescence inverted research microscope (OLYMPUS, Europe). Photographed images were analyzed by means of the *analySIS 3.2* image-computer program (OLYMPUS, Budapest).

3.10. Scanning electron microscopy

The amyloid treated SH-SY5Y cells were washed twice with serum free medium (37 °C) and fixed for 1 h in 1% paraformaldehyde on glass coverslips. Cells were washed with PBS (phosphate buffered saline), and dehydrated by successive incubations in 10, 30, 50, 70 and 90% ethanol for 10 min, followed by the incubation in 100% ethanol for 5 min 3 times, and in acetone for 30 min. Dehydration was completed by drying the coverslips in a desiccating chamber. Coverslips were sputter-coated with gold/palladium and imaged using a HITACHI S2400 scanning electron microscope.

3.11. Measurement of the time-dependent binding of aggregated Aβ on the cells

After a definite duration (0, 2, 5, 10, 20, 30 on 60 min) of treatment of differentiated cells with aggregated Aβ 1-42 (10 μM solution) in a 24-well plate, the well content (1 ml) was removed and the wells were washed twice with PBS (1 ml/well, 37 °C). The residual aggregated Aβ-cell complex was suspended in 0.5 ml of PBS (pH=7.4, 37 °C) and 10 μl CR (0.5 mM solution) was added to each probe and the mixture was left for 10 min. In order to remove unbound CR, solutions were centrifuged twice for 10 min at 5000 and 8000 g. The supernatant was removed carefully with a pipette and the sediment was resuspended in PBS (0.5 ml/probe). The CR content of the cell-Aβ suspension was measured photometrically at 492 nm (free CR) and 550 nm (bound CR) with an ELISA plate reader (Klunk *et al.*, 1999). The CR content of the suspension correlates to the amount of Aβ bound to the cell surface.

3.12. Immunocytochemistry

3.12.1 Hyperphosphorylated tau protein identification: differentiated neuroblastoma cells were incubated with the following peptides: **a.** 10 μM aggregated Aβ 1-42; **b.** a mixture of 10 μM aggregated Aβ 1-42 and 50 μM LPYFDn; **c.** 10 μM aggregated Aβ 1-42 and 50 μM pGn in a 24-well plate for 24 hour. The cells were fixed at room temperature by successive applications of 1% paraformaldehyde (10 min), 100% methanol (1 min) and again 1% paraformaldehyde (20 min). The wells were washed twice with 2% BSA/PBS (2 x 20 min) and PBS (1 ml/well). The polyclonal antibody pSer^{396} Anti-Phospho-Tau developed in rabbit (1:100 dilution) was added to the fixed cells, and the mixture was incubated overnight. The primary antibody was visualized by labeling with FITC Conjugate-Goat Anti-Rabbit IgG, used at a dilution of 1:60 for 4 hours. Controls (lacking primary antibody) had no detectable fluorescence. The primary antibody was tested for the positive activity of Phosphatase Inhibitor Cocktail 2 (a 1:25 dilution in cell culture medium with 2% FBS) via the hyperphosphorylated tau accumulation in the cells. Digital images were photographed (Fig. 19) and analyzed (for details see Image Analysis).

3.12.2 Extracellular Aβ 1-42 identification: differentiated neuroblastoma cells were treated with the aggregated Aβ 1-42 (10 μM) in a 24-well plate for one hour. The wells were washed twice with cell culture medium (37°C). The cells were fixed at room temperature by successive applications of 4% formaldehyde (30 min). The wells

were washed twice with 2% BSA/PBS (2 x 20 min) and PBS (1 ml/well). The mouse monoclonal Anti-Human Amyloid Beta Protein (clone: 4G8; 1:100 dilution) was added to the fixed cells, and the mixture was incubated overnight. The primary antibody was visualized by labeling with FITC Conjugate-Anti-Mouse IgG, used at a dilution of 1:100 for 2 hours. Controls (lacking primary antibody) had no detectable fluorescence. Digital images were photographed (Fig. 18.A.B.) and analyzed.

3.12.3 MAP2 (microtubule-associated protein 2) identification: differentiated neuroblastoma cells were treated with aggregated Aβ 1-42 (10 μM) in a 24-well plate for 24 hours. The cells were fixed at room temperature by successive applications of 4% formaldehyde (30 min). After fixation the cells were treated with 0.1 % detergent, Triton 100X (10 min). The wells were washed three times with 2% BSA/PBS (3 x 20 min) and PBS (1 ml/well). The monoclonal Anti-MAP2 (2a+2b) antibody produced in mouse (1:200 dilution) was added to the fixed cells, and the mixture was incubated overnight. The primary antibody was visualized by labeling with FITC Conjugate-Anti-Mouse IgG, used at a dilution of 1:100 for 4 hours. Controls (lacking primary antibody) had no detectable fluorescence. Digital images were photographed (Fig. 18.C.D.) and analyzed (for details see *Morphological evaluation*).

3.13. Neutral Red assay

The active uptake of a hydrophilic dye was measured by the method of Loske *et al.* (1998).

3.14. Congo red (CR) aggregation assay

A modification of the method of Klunk *et al.* (1999) was used. The aggregated Aβ 1-42 (10 μM) was incubated alone and in other experiments with the different pentapeptide amides (LPYFDn or pGn, at 50 μM final concentration) in 1 ml of PBS, pH=7.4, at 37 °C. After incubation (aggregation) periods of 5 and 24 hours, 10 μl of CR (0.5 mM) was added to each tube (1 ml) and the mixture was incubated for 10 min. Solutions (1 ml) were centrifuged twice for 10 min at 5000 and 8000 g. The supernatant was removed carefully with a pipet and the sediment was resuspended in PBS (0.5 ml/sample). The CR content of the aggregated peptide-

CR suspensions was measured photometrically at 492 nm (free CR) and 550 nm (bound CR) with an ELISA plate reader.

3.15. Oxidative stress measuring

3.15.1 Superoxid level measurement: A modification of the method of Misonou *et al.* (2000) was used. Differentiated neuroblastoma cells were incubated with the following peptides: **a.** 10 μM aggregated Aβ 1-42; **b.** a mixture of 10 μM aggregated Aβ 1-42 and 50 μM LPYFDn in a 24-well plate for 1-5 hours. The superoxid generator paraquat (1 mM) was the positive control, then the cells were fixed at room temperature by successive applications of 1% paraformaldehyde (10 min), 100% methanol (1 min) and again 1% paraformaldehyde (20 min). The wells were washed twice with PBS (1 ml/well). Cells were incubated for 30 min at room temperature with dihydro-ethidine (DHE; 5 μM) in PBS. Digital images were photographed (Fig. 22) and analyzed (see *Image analysis*).

3.15.2 Lipid peroxidation measurement: A modification of the method of Pap *et al.* (1999) was used. Differentiated neuroblastoma cells were treated with 10 μM aggregated Aβ 1-42 in a 24-well plate for 0 or 3 hours. The hydrogen-peroxid (H_2O_2; 100 μM) was the positive control. After treatment, time cells were incubated for 20 min at 37°C with C11-BODIPY$^{581/591}$ (1 μM) in growth medium. Digital images were photographed (Fig. 23) and analyzed (see *Image analysis*).

3.16. Intracellular calcium level ($[Ca^{2+}]_i$) measurement

A modification of the method of Laskay *et al.* (1997) was used. Differentiated neuroblastoma cells were treated with 0.02 % Pluronic acid (5 min), washed twice with culture medium, 5 μM Fura-2 AM for 30 min at 37 °C. For treatment, the following peptides were used: **a.** 10 μM aggregated Aβ 1-42; **b.** a mixture of 10 μM aggregated Aβ 1-42 and 50 μM LPYFDn in a 24-well plate for 300 sec. Images were taken with a F-View camera (with CCD arrays of 1300 x 1030 pixels). These cameras were interlocked with the imaging system of an OLYMPUS IX71 inverted research fluorescence microscope (640x). The objective table of the microscope could be heated up to 37 °C. After the descending line of Aβ effect the cell activity was tested for the positive effect of Ionomycin (30 μM) on the intracellular calcium level.

3.17. TEM-experiments

Aβ 1-42 was dissolved to a concentration of 115 μM in distilled water by constant pipetting for 2 min at 4 °C, followed by a sonication for 10 minutes. Then the solution was diluted to a final concentration of 10 μM and incubated for 24 hours at 37 °C. According to the method of Walsh *et al.*, 10 μl droplet of the peptide solution was adsorbed to a carbon-coated copper grid (Electron Microscopy Sciences, Washington, PA) for 2 min. Then, the droplet was replaced with an equal volume of 0.5% (v/v) glutaraldehyde solution and was incubated for an additional 1 min. Then, the grid was washed with three droplets of water, followed by an incubation of a 10 μl droplet of 2% (w/v) uranyl acetate solution for 2 min. Specimens were studied using a Philips CM 10 transmission electron microscope at 100 kV, routinely at magnifications of ×46,000 and ×64,000.

3.18. Image analysis

Images were taken with a 12-bit digital F-View camera (with CCD arrays of 1300 x 1030 pixels) and a DP 70 color camera. These cameras were interlocked with the imaging system of an OLYMPUS IX71 inverted research fluorescence microscope (640x). Five images for each peptide and peptide mixture (see MTT bioassay) at different treatment durations (minutes or hours) were subjected to histogram analysis with the *analySIS 3.2* and DP 70-BSW 01.01. image-computer program from OLYMPUS (Europe). Five intensity bins were summed for each peptide after each treatment time period to obtain the graphs.

3.19. Statistical analysis

ANOVA Post Hoc Test and subsequently Bonferroni and Dunnett t (2-sided) were used for statistical calculation with SPSS 9.0 for Windows. Neurotoxicity measurement experiments were performed triplicated, unless specified. Each measurement contained 7 parallel treatments (wells). When the differences were significant at $p \leq 0.01$ the values were considered statistically significant. The differences were compared to untreated control values.

4. Results

4.1. Differentiation and characterization of the SH-SY5Y human neuroblastoma cell culture

In the modelling of the AD specific neurodegeneration and neuroprotection in cell cultures, we used different types of cells (Fig. 2): non differentiated and/or differentiated human derived SH-SY5Y neuroblastoma cells and primary neurocyte cultures from rat brain. The fastness and simplicity were the main focuses in the methodologies. The differentiated neuroblastoma cells have relatively long neurites (aprox. 270 μm), similarly to the primary neurons (Fig. 5). Treatment of SH-SY5Y cells with RA (10 μM) dissolved in DMSO (with 0.5 % final concentration in the medium) results in the formation of differentiated SH-SY5Y cells. These cells display only a slight [^{3}H]-thymidine incorporation increase (Figure 2) and a minor protein content change in response to the mitogenic effect of ConA, similarly as for the primary neurocyte culture (Figure 5). In contrast, the non-differentiated cells exhibit a extremely increased [^{3}H]-thymidine incorporation and protein content after the addition of ConA. The response of the differentiated SH-SY5Y cells to the mitogenic substance is time-dependent: 8 to 10 days of treatment with RA seems to be enough for appropriate differentiation. Cell culture medium does not contain DMSO during Aβ treatment, therefore, the membrane structure is not disturbed by the presence of any organic solvents.

Figure 6 demonstrates that the cell number in a differentiated SH-SY5Y culture becomes stabilized in 6-8 days. In neurotoxicity studies, no correction (e.g calculating of the results to protein content) is necessary, if differentiated cells with a constant cell number are used.

Aggregating Aβ peptides induce neurodegeneration in SH-SY5Y neuroblastoma cells (Lambert *et al.*, 1994). Figure 8 illustrates the neurotoxic effects of Aβ 1-42 on SH-SY5Y and differentiated SH-SY5Y cells (8-day-differentiation). While the non-differentiated SH-SY5Y cell number was increased by 70% during a 2-day treatment, the differentiated cell culture displayed a constant cell number (control experiments). As a consequence, no correction is needed when Aβ 1-42 neurotoxicity is measured with the MTT assay on differentiated SH-SY5Y cells. On the other hand, in work with SH-SY5Y cells, the redox activities (OD of soluble formazan) should be corrected by calculation according to the starting cell number (100%).

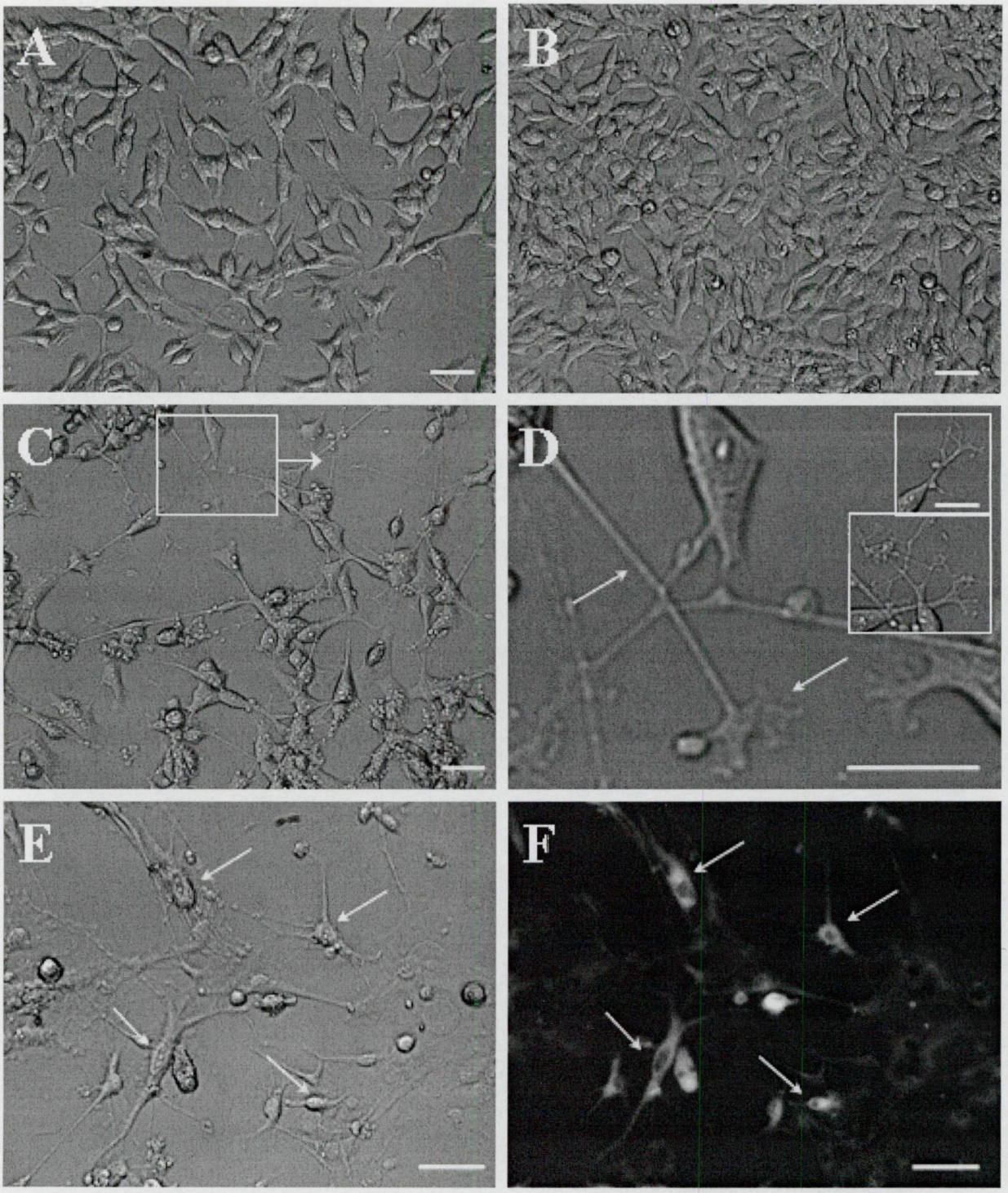


Fig. 2. *Different cell cultures. A) Non-differentiated SH-SY5Y neuroblastoma cells; B) Differentiated SH-SY5Y neuroblastoma cells in confluent monolayer form; C) Differentiated SH-SY5Y neuroblastoma cells in sparsely proliferated form; D) Neurites and neurit terminates of SH-SY5Y neuroblastoma cells (arrows); E) Rat neurocytes in sparsely form; F) Identification of the neurocytes by anti MAP2 antibody (arrows).*

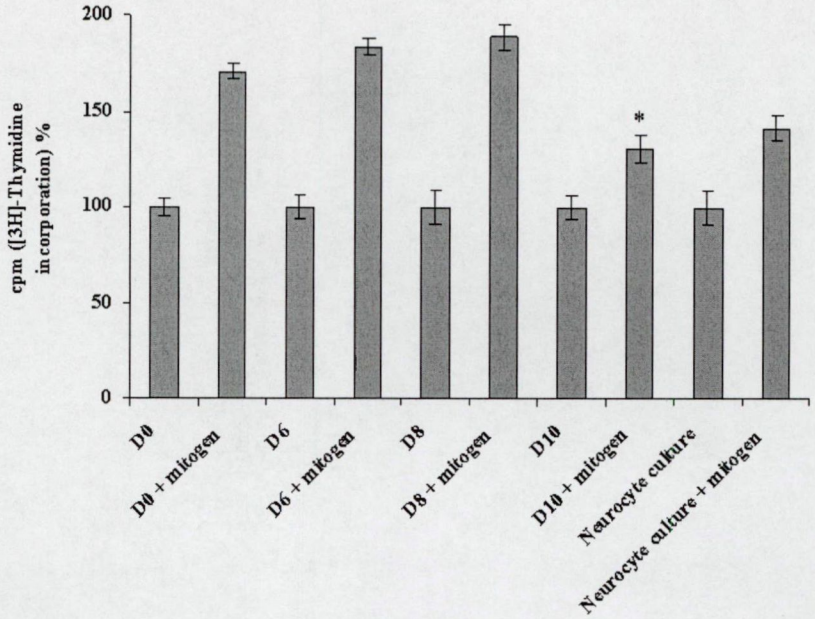


Fig. 3. *The effects of retinoic acid (RA) on cell proliferation detected by thymidine incorporation. (D_0: non-differentiated, control SH-SY5Y cells confluent monolayer; D_6: SH-SY5Y + 10 μM RA, 6-days; D_8: SH-SY5Y + RA, 8-days; D_{10}: SH-SY5Y + RA, 10-days). Mitogen: 0.1 μg/ml ConA for 22 hours, without differentiating agents. *Differences compared to untreated control values (D_0) are significant at p≤0.01, n=21; ANOVA Post Hoc Test, Dunnett t (2-sided).*

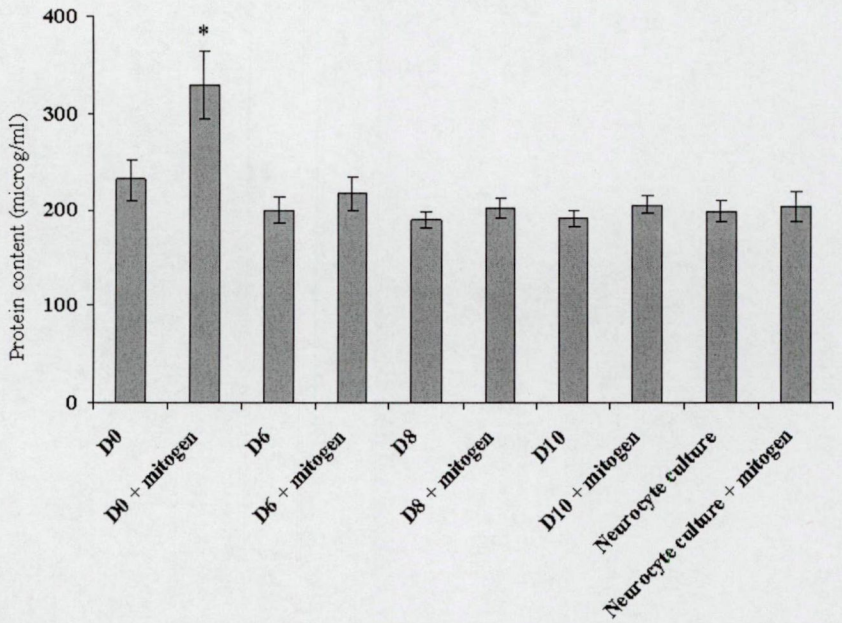


Fig. 4. *The effects of retinoic acid (RA) on cell protein content. (D_0: non-differentiated, control SH-SY5Y cells confluent monolayer; D_6: SH-SY5Y + 10 μM RA, 6- days; D_8: SH-SY5Y + RA, 8-days; D_{10}: SH-SY5Y + RA, 10-days). Mitogen: 0.1 μg/ml ConA for 22 hours, without differentiating agents. * Differences compared to untreated control values (D_0) are significant at p≤0.01, n=21; ANOVA Post Hoc Test, Dunnett t (2-sided).*

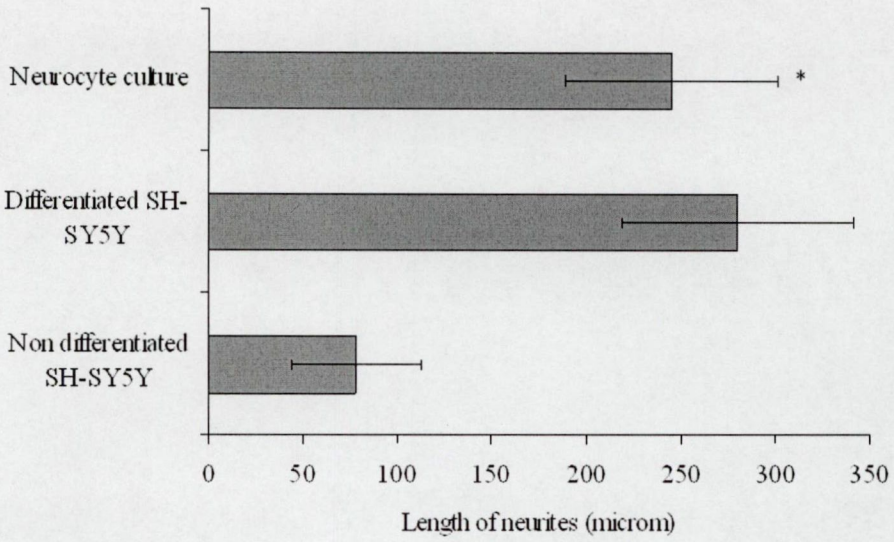


Fig. 5. *Differences in the length of neurites of different cell types in culture. The differentiated SH-SY5Y cell type has the longest neurites. * Differences compared to non-differentiated cells are significant at p≤0.01, n=100; ANOVA Post Hoc Test, Dunnett t (2-sided).*

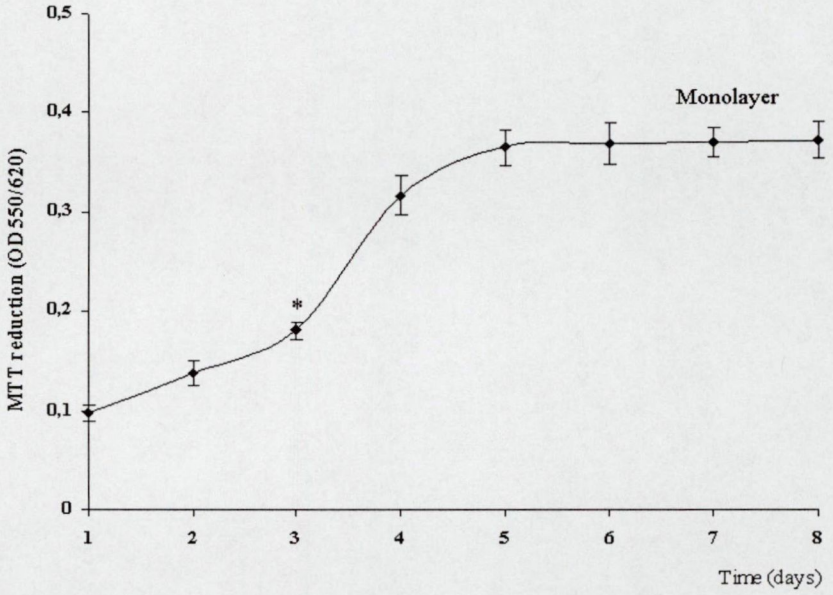


Fig. 6. *Changes in cell proliferation during the first 8-days of the differentiation process, as measured with MTT assay. After treatment with RA, the cell number was increased and in 8 days stabilized. The initial cell number was chosen to be 2.5 x 10^5/ml in order to reach the monolayer state and maximal cell differentiation in the same time. * Differences compared to untreated control values (1-day) are significant at p≤0.01, n=21; ANOVA Post Hoc Test, Dunnett t (2-sided).*

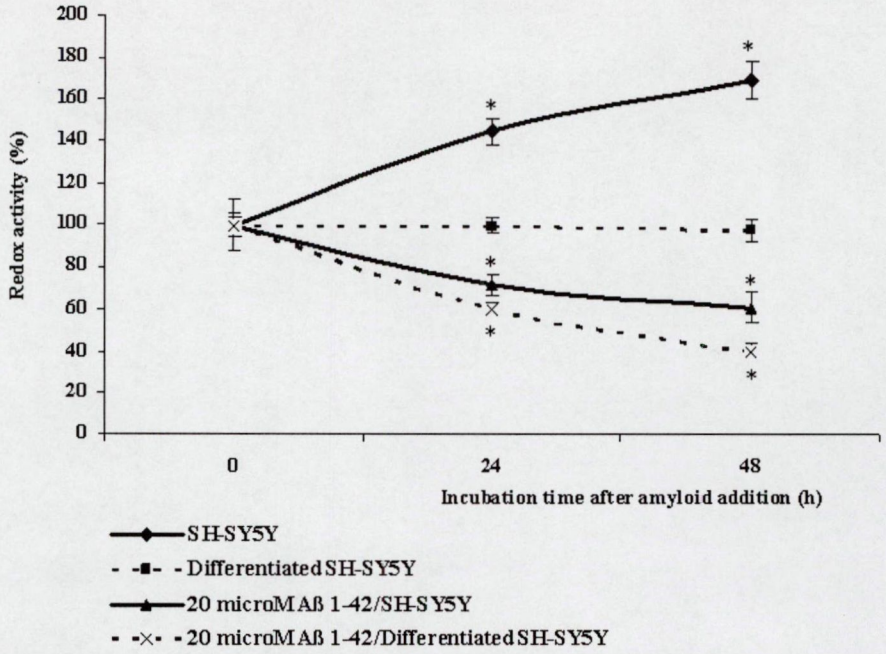


Fig. 7. *MTT assay of non-differentiated (full line) and 8-day-differentiated SH-SY5Y cells (dotted line), without treatment and after treatment for 24 or 48 hours with 20 μM Aβ 1-42. (In consequence of the increasing number of non-differentiated cells, the values are calculated relative to the original cell number). * Differences compared to untreated control values (1 day) are significant at p≤0.01, n=21; ANOVA Post Hoc Test, Dunnett t (2-sided).*

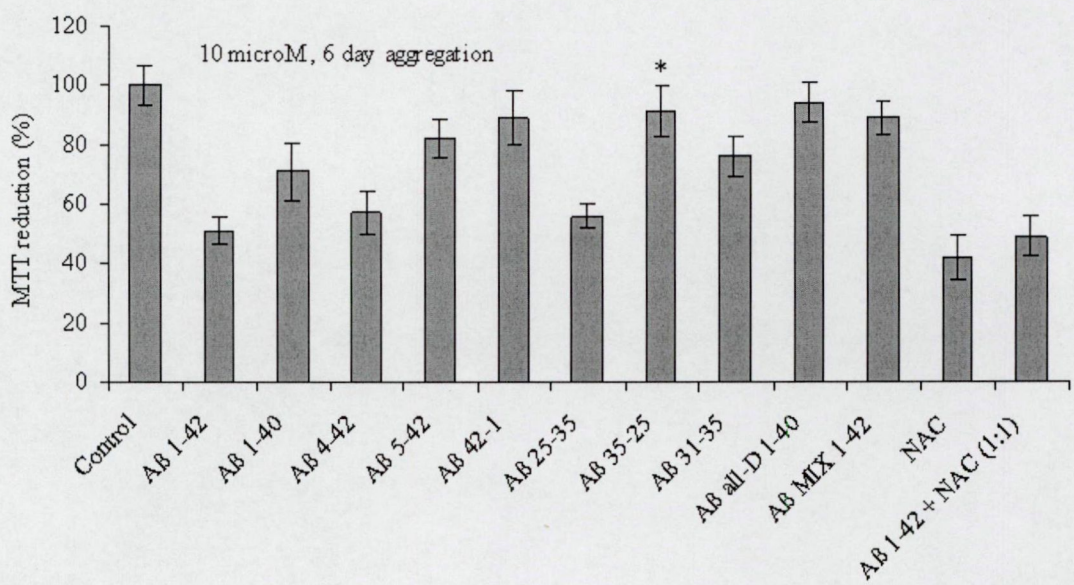


Fig. 8. *Neurotoxic activities for 24 hours of aggregated peptides on differentiated cells, measured with MTT assay. * Differences compared to untreated control values are significant at p≤0.01, n=21; ANOVA Post Hoc Test, Dunnett t (2-sided).*

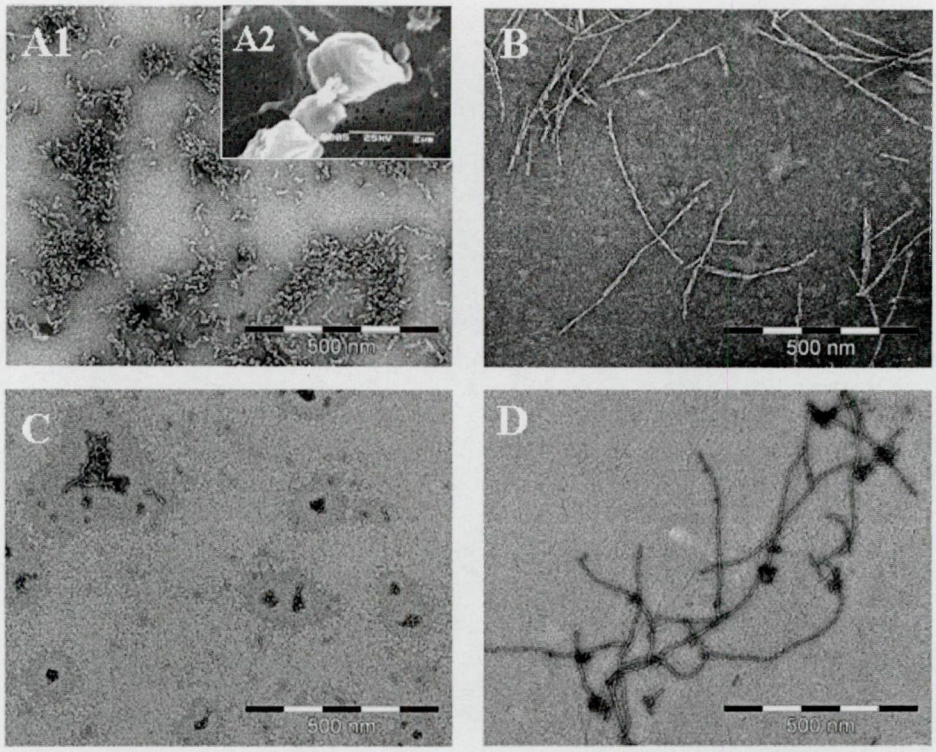


Fig. 9. *Electron micrograph of Aβ 1-42 fibrils formed in a 10 μM peptide solution after 24 hours of incubation at 37 °C (uranyl acetate background): A/1) Aβ 1-42 (1-day aggregation); A/2) aggregated Aβ 1-42 clusters bound to cells (scanning electron microscopy, bar represent 2 μm). According to Congo red staining, the bright spherical clusters are Aβ 1-42 aggregates (light microscopical control); B) Aβ 1-40 (6-day aggregation); C) Aβ 4-42 (8 day aggregation); D) Aβ 5-42 (8- day aggregation).*

4.2. Measuring the neurotoxicity of different aggregated peptides on differentiated SH-SY5Y neuroblastoma cells

The cell biology of Alzheimer`s disease is characterized by neurodegeneration caused by the β-amyloid (Aβ) peptides (Smith et al., 2002). We used differentiated SH-SY5Y cells (8-day-differentiation) to measure the neurotoxic effect of different Aβ-peptides and alpha-synuclein fragments (ex. NAC) in the MTT assay (Figure 9). Aβ 1-42 aggregated rapidly, therefore, it was always aged for 1 day; the other peptides needed a longer (6-8 day) aging time (Fig. 9). 10 μM Aβ 1-42 decreases the redox activity of the cells to 50%. Aβ 1-40 is also neurotoxic at 10 μM, but it causes a smaller decrease of redox activity. The reverse peptide structure (Aβ 42-1) exhibits only a very small tendency to aggregation and shows no significant toxicity. Interestingly, the reverse peptide Aβ 35-25 shows some neurotoxic effects. The applied NAC reveal high tendency to aggregation and high neurotoxicity.

4.3. The neurotoxicity of aggregated Aβ 1-42 is time and dose dependent

We have found that the effect of aggregating Aβ 1-42 to the cell viability (measured with MTT and Neutral Red assay) is time and dose dependent. Ten μM peptide concentration and 24 hours treatment is necessary for 50 % decrease in cell viability. Therefore, we used these two parameters.

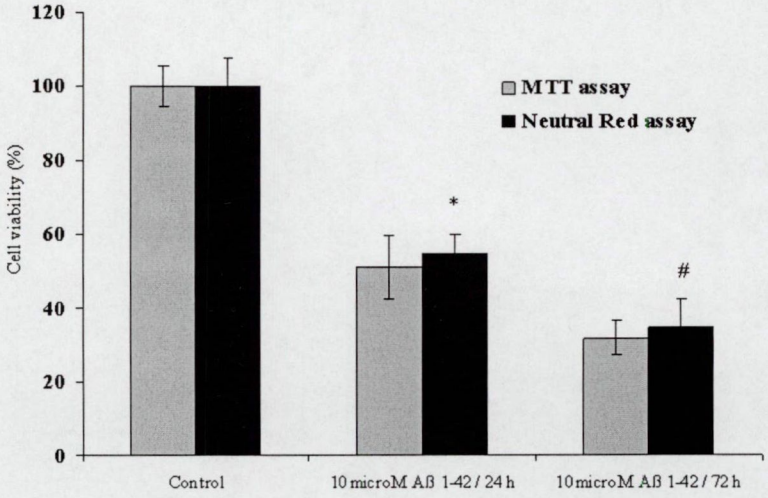


Fig. 10. *The toxic effect of Aβ 1-42 on differentiated SH-SY5Y cells. Neuroblastoma cells were treated for 24 and 72 hours with aggregated Aβ 1-42 (10 μM). The effect of Aβ 1-42 on cell viability (measured with MTT and Neutral red assay) was time dependent. * Differences compared to the untreated control values are significant at a level p≤0.01, n=21. Statistical analysis by ANOVA post hoc test, Bonferroni.*

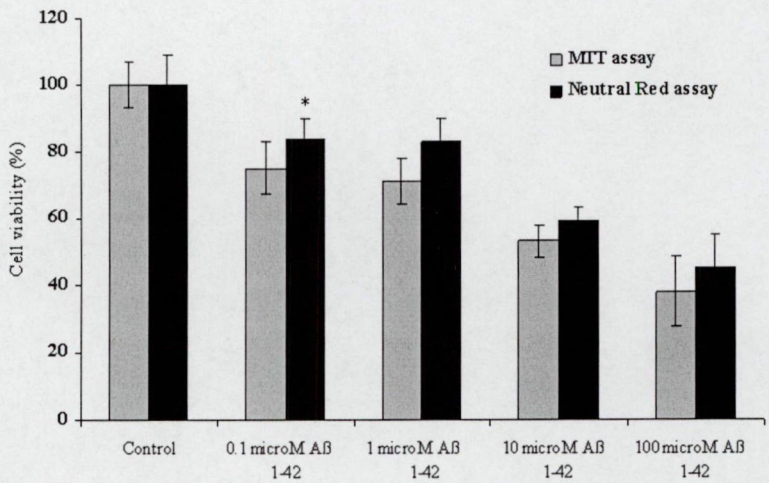


Fig. 11. *The toxic effect of Aβ 1-42 on differentiated SH-SY5Y cells. Neuroblastoma cells were treated with different concentrations of aggregated Aβ 1-42 (0.1, 1, 10 and 100 μM for 24 hours). The effect of Aβ 1-42 on cell viability (measured with MTT and Neutral red assay) was dose dependent. * Differences compared to the untreated control values are significant at a level p≤0.01, n=21. Statistical analysis by ANOVA post hoc test, Bonferroni.*

4.4. Exogenous Aβ 1-42 induces in vitro neurite degeneration in diff. SH-SY5Y cells

In vitro experiments serve to model the pathophysiological Aβ accumulation on the surfaces of the neurons. The SH-SY5Y neuroblastoma cells possess highly developed neurites and exhibit high sensitivity against Aβ. Morphological changes could be observed on the cells during Aβ 1-42 treatment (Fig. 12). Aggregated Aβ 1-42 clusters bound strongly to the cells: diffusion was unable to remove them from the cells. Aggregated Aβ 1-42 bound mainly to the lamellipodia and the edge of the perikaryon. Two main morphological changes were observed during treatment. The neurites of treated cells became rougher and thinner than those of the untreated cells. This was a time-dependent process, very characteristic within 1 h (Fig. 13). The vanadate containing phosphatase inhibitor cocktail 2 (in 1:25 dilution) was the positive control. Contraction (rounding-off) of the perikaryon was the other main morphological change. This was also a time-dependent process which took place within 2 hours. The morphological changes, which occured during Aβ treatment, were examined more exactly by scanning electron microscopy (Fig. 9/A2). This confirmed that the Aβ 1-42 clusters bind to the cell surface. After 24 hours of treatment with 10 μM Aβ 1-42, the microtubular system was disintegrated (Fig. 18/C and 18/D).

No significant change in cell morphology was observed when Aβ 1-42 was co-aggregated with the pentapeptide LPYFDn for 24 hours before treatment (Fig. 14).

4.5. Exogenous Aβ 1-42 decreases the cell viability in differentiated SH-SY5Y cells

The MTT (redox state) and Neutral Red (active membrane uptake) assays provide information about the viability of the cells. Figure 16 demonstrates that aggregated Aβ 1-42 significantly decreases the redox potential of the cells (MTT-assay; Datki *et al.*, 2003). This effect of the Aβ 1-42 is time (Fig. 10) and dose (Fig. 11) dependent. Vanadate (PIC2) also decreases the cell viability to 40%. The pentapeptide LPYFDn itself is not toxic and at 50 μM prevents the toxic effect of Aβ 1-42 aggregates almost perfectly. In a similar experiment, pGn has practically no protective effect.

Recovery of the cells after Aβ 1-42 treatment was also studied by using different duration of treatments (Fig. 16). Although the cell culture medium, containing Aβ 1-42 aggregates, were thoroughly removed from the cells; a short (2-min) treatment of the cells resulted in a significant decrease of the cell viability. (Extensive washing can not remove the membrane-bound Aβ aggregates perfectly, see Fig. 9/A2).

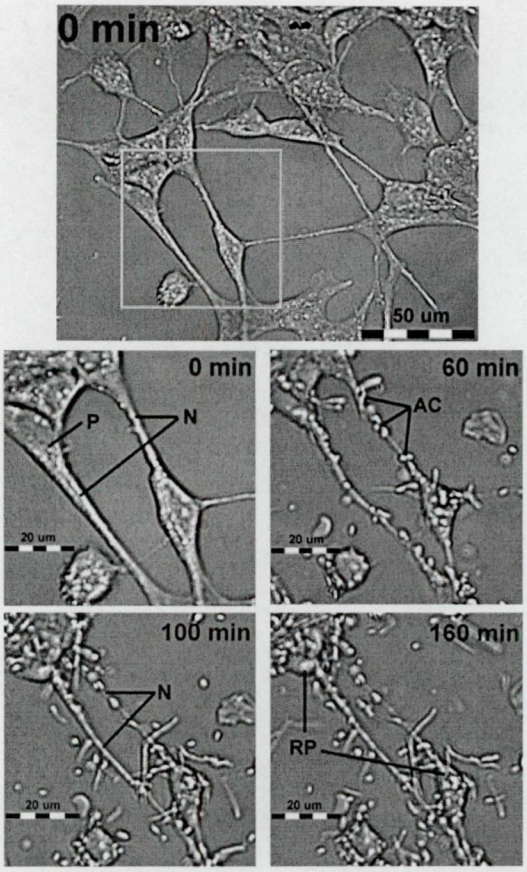


Fig. 12. *Cells were incubated with 10 μM aggregated Aβ 1-42 for 160 min. Pictures were photographed with a light-fluorescence inverted research microscope equipped with a CCD camera at 460x magnification. The cell morphology changed dramatically (AC = Aβ cluster; N = neurite; P = perikaryon; RP = rounding perikaryon).*

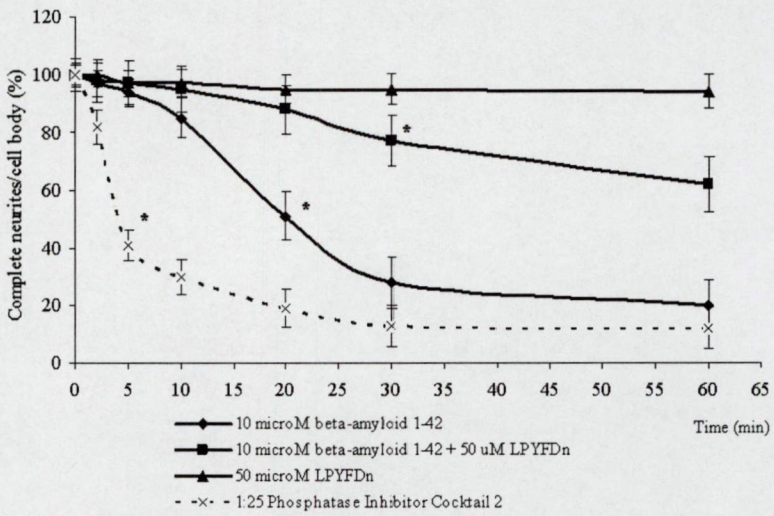


Fig. 13. *Decrease in the number of neurites in differentiated SH-SY5Y cells caused by aggregated Aβ 1-42 (10 μM). The vanadate containing phosphatase inhibitor cocktail 2 (in 1:25 dilution) was the positive control. Aβ 1-42 causes neurite degeneration in the first 20 min after treatment. The pentapeptide LPYFDn significantly prevent the*

*neurite degeneration caused by the aggregated Aβ 1-42. * The differences in the number of the complete neurites per cell body, compared to the LPYFDn alone are significant at a level p≤0.01; n=100 in the ANOVA post hoc test, Bonferroni.*

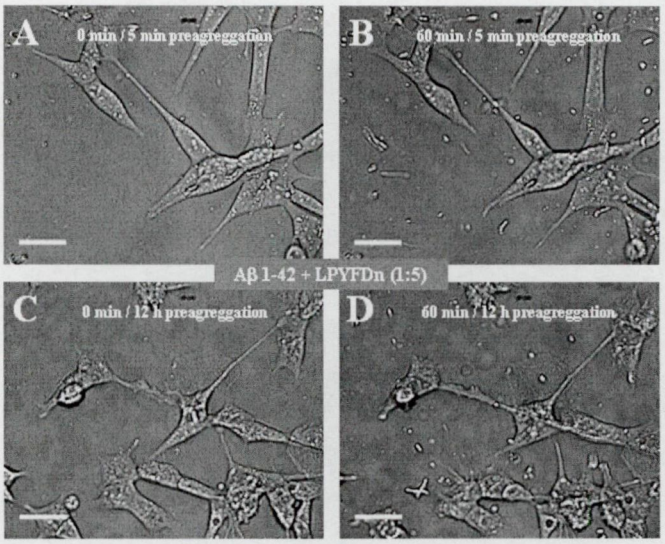


Fig. 14. *SH-SY5Y cells were incubated with 10 μM Aβ 1-42 together with 50 μM LPYFDn pentapeptide for 60 min. These two peptides were pre-aggregated together for 5 min and/or 12 hours. Pictures were photographed with a light-fluorescence inverted research microscope equipped with a CCD camera at 460x magnification (bar represents 20 μm). The cell morphology did not change dramatically.*

The longer the duration of Aβ treatment, the lower the cell viability. The results in Fig. 16 reveal that treatment with Aβ 1-42 for 2 min causes irreversible changes in the cells; even recovery for 24 hours was unable to completely restore cell viability.

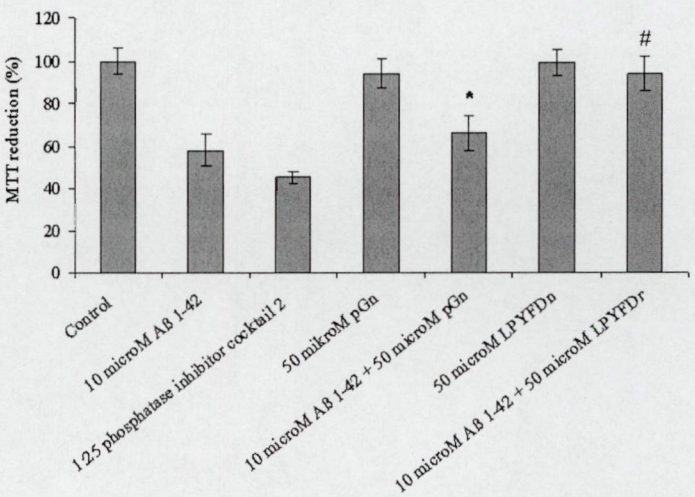


Fig. 15. *The effect of Aβ 1-42 and short pentapeptides on cell viability. Cell viability was detected by means of the MTT assay on SH-SY5Y cells. The aggregated Aβ 1-42 (10 μM) significantly decreased the cellular redox*

*potential. The vanadate-containing phosphatase inhibitor cocktail 2 (1:25 dilution) was also toxic. The pentapeptide LPYFDn is not toxic and at 50 μM prevents the toxic effect of Aβ 1-42. pGn can not prevent the decrease in the redox potential caused by Aβ 1-42. * Differences compared to the untreated control values are significant at a level p≤0.01, n=21. # Differences compared to the Aβ 1-42 values are significant at a level p≤0.01; n=21. Statistical analysis by ANOVA post hoc test, Bonferroni.*

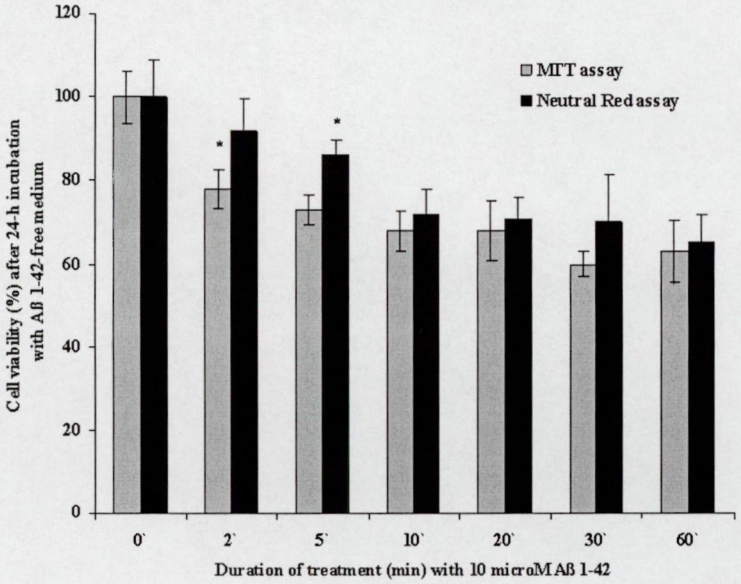


Fig. 16. *The toxic effect of Aβ 1-42 on differentiated SH-SY5Y cells after a short treatment and recovery for 24 hours. Neuroblastoma cells were treated with aggregated Aβ 1-42 (10 μM) from 0 to 60 min. After the treatment, the cell culture medium, containing Aβ, was removed and the cells were washed with new cell culture medium. The living cells were incubated for recovery in culture medium (24 h, 37 °C and 5% CO_2 without Aβ). Cell viability (measured with MTT and Neutral red assay) decreased irreversibly in the cells after Aβ 1-42 treatment for 2 to 60 min. * Differences compared to the untreated control values are significant at a level p≤0.01, n=21. Statistical analysis by ANOVA post hoc test, Bonferroni.*

4.6. The binding of exogenous Aβ 1-42 to differentiated SH-SY5Y cells is a time-dependent process

The optical densities (ODs) measured at 550 nm (Aβ-bound CR) correlate with the amount of Aβ bound to the cell membranes (Klunk *et al.*, 1999). Figure 18 reveals that the binding of Aβ 1-42 aggregates (10 μM) to the cell membranes depends on the duration of time. The binding capacity of the cells is saturable, reaching its limit in 60 min. Neurites are the critical part of the neuroblastoma cells (Fig. 18/A and 18/B). After 24 hours treatment with 10 μM Aβ 1-42, the microtubular system was disintegrated (Fig. 18/C and 18/D). The visualized microtubule-associated protein 2 (MAP2) is specific to the neurons and neuroblastoma cells.

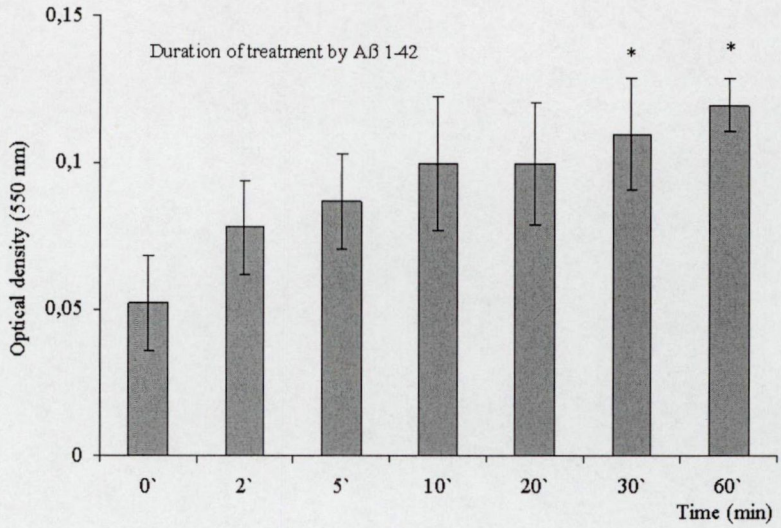


Fig. 17. *The time dependence of Aβ 1-42 binding to differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were treated from 0 to 60 min with Aβ 1-42 (10 μM) aggregated for 1 hour. The supernatant was removed from each well after treatment; the cells were fixed with 1% paraformaldehyde and stained with 10 μM Congo red (CR). The CR-stained Aβ content increased time-dependently. The optical density (OD) of amyloid-bound CR (550 nm; Klunk et al., 1999) reflects the amount of Aβ adsorbed on the cell membrane surface. * OD differences (compared to the values at 0 and 2 min) are significant at $p \leq 0.01$, $n=10$ (statistical analysis: ANOVA post hoc test, Bonferroni).*

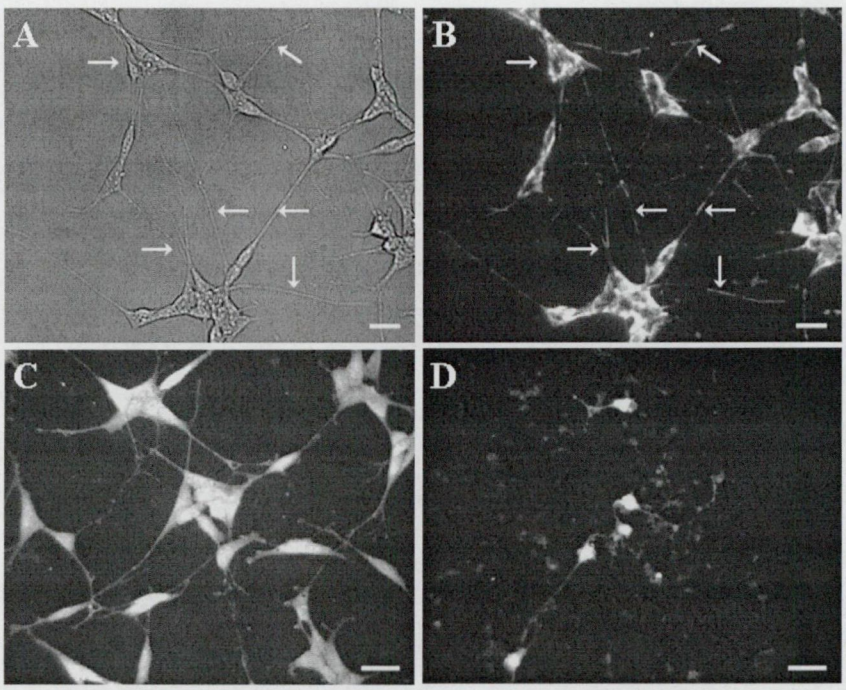


Fig. 18. *The immunocytochemistry of differentiated SH-SY5Y neuroblastoma cells. A) and B) Cells were incubated with 10 μM aggregated Aβ 1-42 for 1 hour. The Aβ 1-42 (identified with anti-amyloid antibody) binds to the surface of the cells (cell bodies, neurites; arrows). C) MAP2 immunocytochemistry. D) Cells were incubated with*

10 μM aggregated Aβ 1-42 for 24 hours. The cell morphology changed dramatically, the microtubule system was deorganized. Pictures were photographed with a light-fluorescence inverted research microscope equipped with a CCD camera at 460x magnification (bar represents 20 μm).

4.7. Exogenous Aβ 1-42 increases tau hyperphosphorylation in differentiated SH-SY5Y cells

Aβ 1-42 is known to cause the significant hyperphosphorylation of tau proteins (Fig. 19; Mookherjee *et al.*, 2001). Figure 20 shows that this process reaches its maximal value in the Aβ-treated cells within 30 min. (When the phosphatase activity was inhibited with a vanadate-containing cocktail, the amount of hyperphosphorylated tau proteins increased: the shift of the phosphorylation-dephosphorylation equilibrium resulted in the formation of hyperphosphorylated tau proteins. Vanadate ion is analogous with phosphate ion possessing a very similar structure and inhibiting the activity of phosphatases in competitive way).

The pentapeptide LPYFDn decreased tau hyperphosphorylation (Fig. 20, co-treatment with Aβ 1-42). In another experiment, co-treatment of the cells with Aβ 1-42 and pGn resulted in the same increase of hyperphosphorylation as that due to Aβ 1-42 alone (not shown in Fig. 20).

Fig. 21 shows that the first 10 min of Aβ 1-42 treatment causes reversible intracellular tau hyperphosphorylation: the washing-out of unbound Aβ enables the cells to recover almost completely. However, the amount of hyperphosphorylated tau proteins remained high after treatment with Aβ 1-42 for 20, 30 or 60 min, and after washing and recovery for 24 hours (48 and 72 hours periods for recovery showed practically no difference compared to the results after 24 hours). This shows that the biochemical processes initiated by Aβ treatment in neuronal cells become irreversible after 10 min.

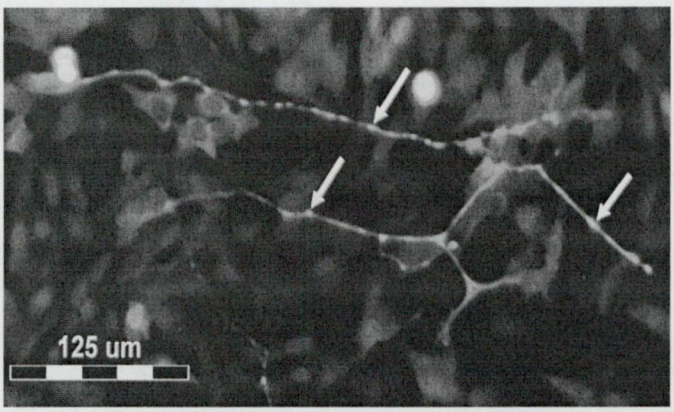


Fig. 19. *The accumulation of hyperphosphorylated tau protein in differentiated SH-SY5Y cells (arrows) labelled with pSer396 polyclonal antibody after 30 min of Aβ 1-42 treatment. Primary antibody was visualized by labelling with FITC-conjugated secondary antibody. Digital images were photographed.*

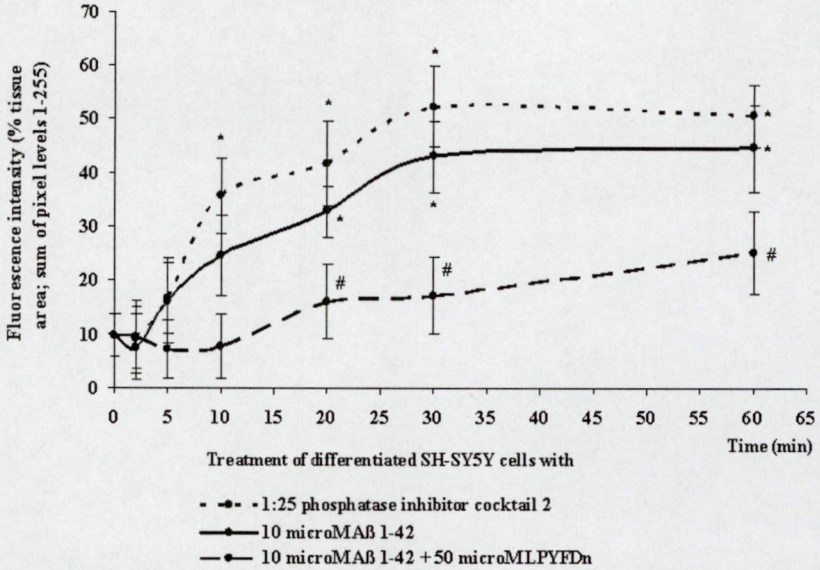


Fig. 20. *Increase in tau hyperphosphorylation in differentiated SH-SY5Y cells caused by aggregated Aβ 1-42. Tau hyperphosphorylation was detected with FITC-labeled antibodies. Aggregated Aβ 1-42 (10 μM) increased the fluorescence intensity significantly during the first 20 min after Aβ treatment. The vanadate containing phosphatase inhibitor cocktail 2 (a 1:25 dilution) was the positive control: the inhibition of phosphatases (and the activation of kinases by Aβ 1-42) causes tau hyperphosphorylation in the first 10 min after treatment. The pentapeptide LPYFDn significantly inhibits tau hyperphosphorylation caused by the aggregated Aβ 1-42. * The differences in the fluorescence intensity compared to the untreated control values (0 min) are significant at a level p≤0.01. # The Fluorescence intensity differences compared to the Aβ 1-42 values are significant at a level p≤0.01; n=5 (five histograms for each peptide and each treatment time were summed to obtain the graph above) in the ANOVA post hoc test, Bonferroni.*

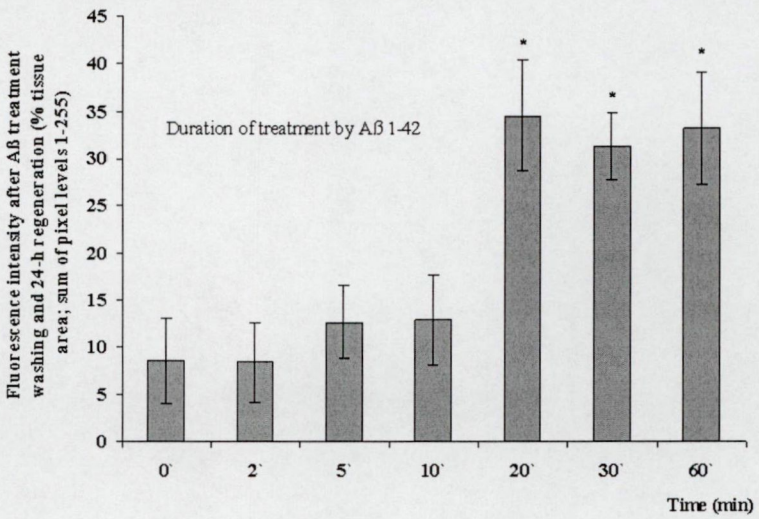


Fig. 21. *The amount of hyperphosphorylated tau in differentiated SH-SY5Y cells after a short treatment with Aβ 1-42 and recovery for 24 hours. The neuroblastoma cells were treated with aggregated Aβ 1-42 (10 μM) from 0 to 60 min. After the treatment, the cell culture medium, containing Aβ, was removed and the cells were washed with*

*cell culture medium. The living cells were incubated in culture medium for recovery (24 h, 37 °C and 5 % CO_2 without Aβ). The fluorescence intensity (measured by immunocytochemistry with FITC-labeled antibody) suddenly increased between the Aβ 1-42 treatment periods of 10 and 20 min. The fluorescence intensities are equivalent to the quantities of hyperphosphorylated tau proteins in the Aβ-treated cells. * Differences compared to the untreated control values (0 min) are significant at a level p≤0.01; n=5 (five histograms for each peptide and each treatment time were summed to obtain the graph above) in the ANOVA post hoc test, Bonferroni.*

4.8. Exogenous Aβ 1-42 induces oxidative stress in differentiated SH-SY5Y cells

Aβ 1-42 is known for causing oxidative stress in human neurons (Cutler *et al.*, 2004). Figure 23 shows that this process reaches its maximal value in the Aβ-treated cells within 3 hours. (The paraquat was the positive control for the superoxid generation).

The pentapeptide LPYFDn decreased superoxid generation (Fig. 22, co-treatment with Aβ 1-42). In another experiment, co-treatment of the cells with Aβ 1-42 and pGn resulted in the same extent of increase of superoxid level as that due to Aβ 1-42 alone (not shown in Fig. 22). Fig. 23 shows that the lipid peroxidation increased after 3 hours of Aβ 1-42 treatment (oxidated form of the fluorescent dye increased and the non oxidated form decreased).

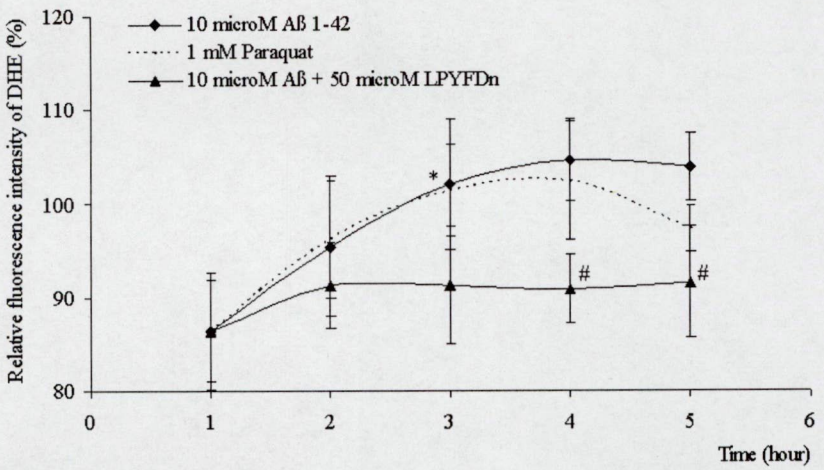


Fig. 22. *Increase in the superoxid ($O_2^{\cdot-}$) content in differentiated SH-SY5Y cells caused by aggregated Aβ 1-42. Oxidative stress was detected with dihydro-ethidine (DHE). Aggregated Aβ 1-42 (10 μM) increased the $O_2^{\cdot-}$ significantly during the 3 hours after Aβ treatment. The superoxid generator paraquat (1 mM) was the positive control. The pentapeptide LPYFDn significantly prevents the oxidative stress caused by the aggregated Aβ 1-42. * The differences in the fluorescence intensity compared with the starting values (1 hour) are significant at a level p≤0.01. # The fluorescence intensity differences compared to the Aβ 1-42 values are significant at a level p≤0.01; n=5 (five histograms for each peptide and each treatment time were summed to obtain the graph above) in the ANOVA post hoc test, Bonferroni.*

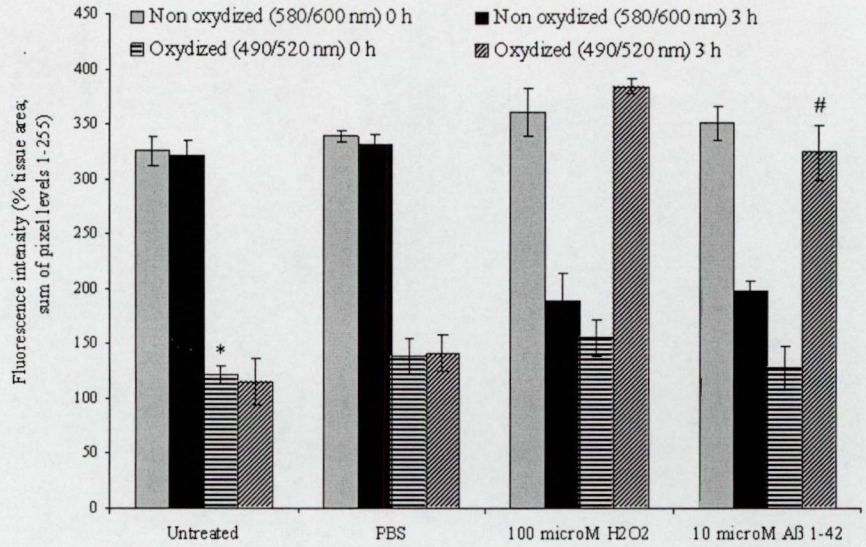


Fig. 23. *Increase in the peroxidated lipid content in differentiated SH-SY5Y cells caused by aggregated Aβ 1-42. Lipid peroxidation was detected with C11-BODIPY$^{581/591}$. Aggregated Aβ 1-42 (10 μM) increased the lipid peroxidation (oxydized form) significantly during the 3 hours after Aβ treatment. Hydrogen-peroxid (H_2O_2; 100 μM) was the positive control. * The differences in the fluorescence intensity compared to the non-oxydized form are significant at a level p≤0.01. # The fluorescence intensity differences compared to the oxidated form (0 hour) values are significant at a level p≤0.01; n=5 (five histograms for each peptide and each treatment time were summed to obtain the graph above) in the ANOVA post hoc test, Bonferroni.*

4.9. Exogenous Aβ 1-42 increases intracellular calcium level in differentiated SH-SY5Y cells

Aβ 1-42 causes calcium influx in human neurons (Pereira *et al.*, 2004). Figure 25 shows that this process reaches its maximal value in the Aβ-treated cells within 50 sec. The amyloid induced calcium signal reached a continuous value. (Ionomicin was the positive control for the calcium influx mechanism).

The pentapeptide LPYFDn inhibited the calcium influx in the differentiated neuroblastoma cells (Fig. 24, co-treatment with Aβ 1-42).

4.10. The inhibitory effect of the Aβ 1-42 on cell attachment

In our experiments, the Aβ 1-42 alone, and the amyloid or fibronectin analog peptides (FRHDSn; GRGDSn) inhibited significantly the cell number bound to the surface of the wells. (The anti-integrin β1 antibody was the positive control). Other amyloid analog peptides (e.g. KLVFFn, RIIGLn or LPYFDn) did not inhibit the cell attachment to the surface of the wells (Fig. 25). This measurement highlighted the important role of adhesion molecules (ex. integrins) in the mechanism of amyloid neurotoxicity.

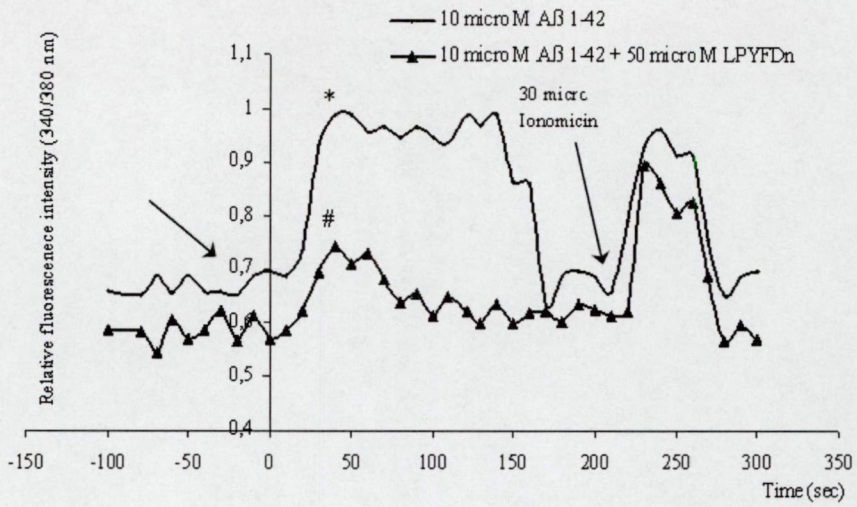


Fig. 24. *Increase in the intracellular calcium level ($[Ca^{2+}]_i$) in differentiated SH-SY5Y cells caused by aggregated Aβ 1-42 (10 μM). $[Ca^{2+}]_i$ was detected with Fura-2 AM (5 μM). Aggregated Aβ 1-42 (10 μM) increased the $[Ca^{2+}]_i$ significantly during the first min after Aβ treatment. Ionomicin (30 μM) was the positive control. The pentapeptide LPYFDn significantly prevented the increase of the $[Ca^{2+}]_i$ caused by the aggregated Aβ 1-42. * The differences in fluorescence intensity compared to the starting values (0 sec) are significant at a level p≤0.01. # The fluorescence intensity differences compared to the Aβ 1-42 values are significant at a level p≤0.01; n=8 in the ANOVA post hoc test, Bonferroni.*

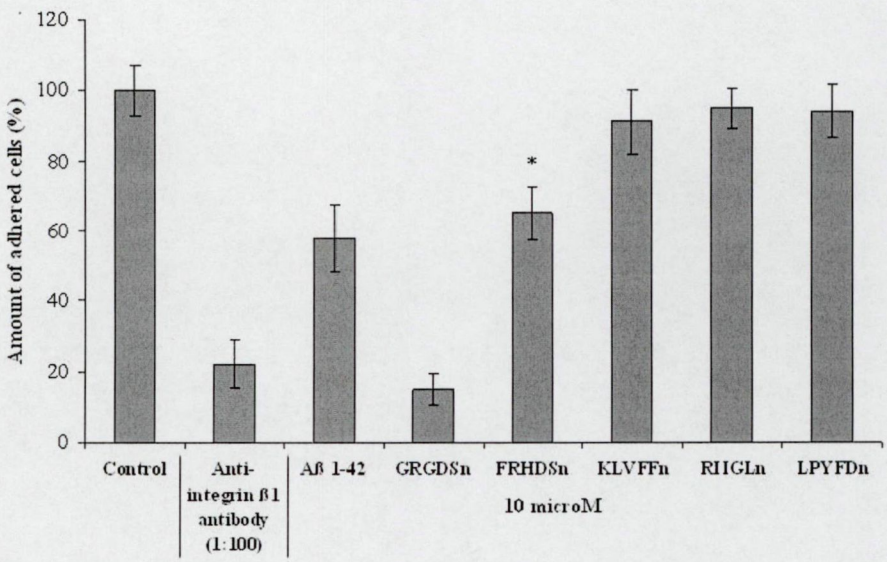


Fig. 25. *The inhibitory effect of the Aβ 1-42 (10 μM) on cell attachment. The anti-integrin β1 antibody (1:100 dilution) was the positive control. Aggregated Aβ 1-42, GRGDSn and FRHDSn pentapeptides inhibited the cell attachment to the 24 well plate surface. The KLVFFn, RIIGLn and the LPYFDn pentapeptides had no negative effect on cell attaching. * Differences compared to the untreated control values are significant at a level p≤0.01, n=21. Statistical analysis by ANOVA post hoc test, Bonferroni.*

4.11. Kinetic studies of interaction between aggregated Aβ 1-42 and LPYFDn

LPYFDn was found to protect neuroblastoma cells against the toxic effect of Aβ 1-42 aggregates. We also studied the possible interaction between Aβ 1-42 and LPYFDn or pGn by using the simple CR assay (Fig. 26) in a cell-free system. It is known that Aβ 1-42 aggregates bind CR (Klunk *et al.*, 1999), forming salt bridges and H-bonds (Carter *et al.*, 1998; Khurana *et al.*, 2001). The amount of CR bound to Aβ can be measured spectrophotometrically (λ_{max} 550 nm). It was found that pGn had no effect on the Aβ-CR complex (Fig. 26). LPYFDn alone cannot be bound to CR. Since incubation of Aβ 1-42 with LPYFDn for 5 h decreased the amount of Aβ-bound CR by 50%, this pentapeptide clearly interacts with Aβ 1-42 aggregates. 24 hours incubation of Aβ 1-42 aggregates with LPYFDn, completely prevented the binding of CR to Aβ 1-42 aggregates.

Figure 28 represents the interaction between Aβ 1-42 and LPYFDn obtained by computational blind docking experiments. [The methodology of blind docking was published in our previous studies (Hetényi *et al.*, 2002a; Hetényi *et al.*, 2002b).] The 18-22 region (VFFAE) of the Aβ 1-42 might be involved in the interaction with the LPYFDn peptide according to the calculations. This finding correlates with the results of Carter *et al.*, where the Aβ 16-22 sequence (KLVFFAE) proved to be active in binding of Congo red. The common active region (VFFAE) of LPYFDn and Congo red is a plausible reason of the decrease of Congo red binding to the amyloid peptide in the presence of LPYFDn.

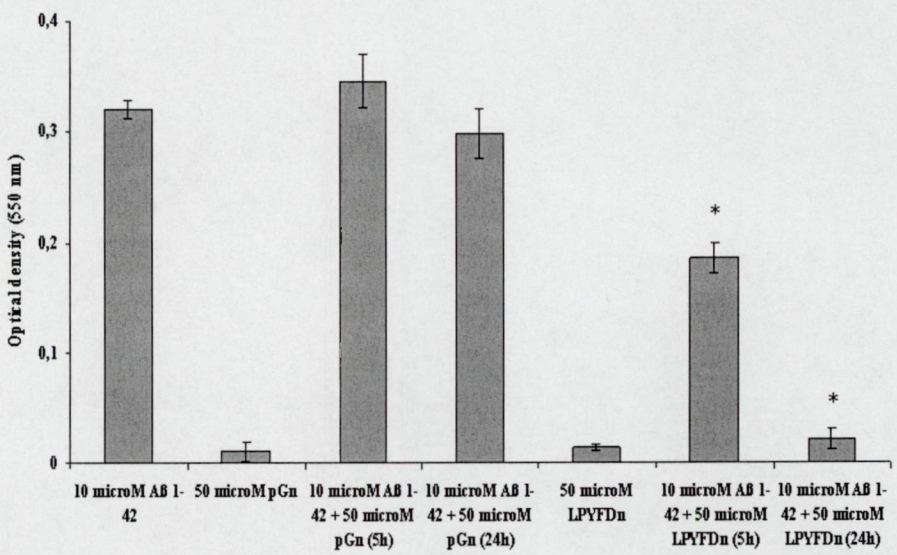


Fig. 26. *The interaction between aggregated Aβ 1-42 and pentapeptide LPYFDn, measured by CR assay. The aggregated Aβ 1-42 (10 μM) was stained by CR (10 μM). The pentapeptide LPYFDn (50 μM) significantly*

*changed the CR binding affinity (incubation time 5 or 24 hours) of the aggregated Aβ 1-42. pGn (50 μM) did not modify the CR binding affinity to the Aβ peptide. *Optical density differences compared to the positive control Aβ 1-42 values are significant at a level p≤0.01, n=5, in ANOVA post hoc test, Bonferroni.*

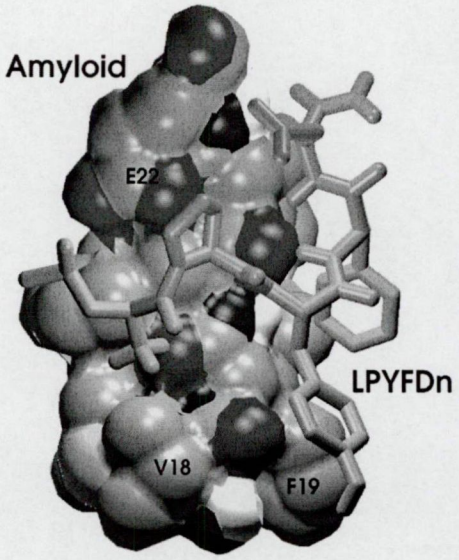


Fig. 27. *The steric model of binding between Aβ 1-42 and LPYFD. The structure of the LPYFDn pentapeptide-amide (sticks) docked on the surface of the Aβ 1-42 molecule. Due to the blind docking study, the V18...E22 sequence of the β-amyloid molecule is involved in the interaction.*

5. Discussion

Our results demonstrated that the MTT assay can be used for neurotoxicity studies of aggregating peptides, and the application of differentiated cells has many advantages. Ba *et al.* (2003) found similar results on using the cell proliferation reagent WST-1 assay for neurotoxicity investigations. Reliable *in vitro* assays are essential for study of the effects of neurotoxic compounds such as β-amyloid peptides. An 8-10 day-treatment of the neuroblastoma cells with RA results in a high differentiation grade which is similar to that for primary cell cultures. Differentiated cells are both morphologically and physiologically very close to living neurons in the brain, therefore the results of neurotoxicity studies are much more reliable with differentiated than with non-differentiated cells. A differentiated SH-SY5Y cell is a good *in vitro* "neuron-model". Correction of the results is not necessary in consequence of the constant cell number in the differentiated cell culture.

We set out to study the early events of neurite degeneration and the intracellular changes caused by Aβ 1-42 aggregates in the differentiated SH-SY5Y neuroblastoma cell line (Fig. 28). We used in all experiments fibrillar Aβ 1-42. Light microscopical examination (Fig. 12) shows that severe morphological changes occur during the first 60 min: Aβ 1-42 clusters bind to the cells and cause the degeneration of the neurites and the contraction (rounding-off) of the perikaryon. The degeneration becomes more severe as time passes. The light microscope proved to be a useful tool for the study of neurodegeneration. Obviously, scanning electron microscopy furnishes more information: Aβ 1-42 forms large spherical clusters with diameters up to 2 μm (Fig. 9/A2), and these clusters bind to the cell surface. Direct measurement of Aβ 1-42 aggregates by using the CR method (Fig. 17) and immunocytochemistry (Fig. 18/A/B) indicated that the amount of cell-bound Aβ 1-42 steadily increased during the first 60 min (Fig. 17). The concentration of CR correlates with the amount of aggregated Aβ 1-42. We suppose that the primary factor, which mediates the effects of Aβ 1-42, is the amount of the peptide bound to the membranes. Further experiments with specific antibodies and quantitative measurement of hyperphosphorylated tau proteins proved that Aβ 1-42 aggregates give rise to a significant increase within a short time (20 min). The amount of hyperphosphorylated tau proteins reaches its maximum in 30 min (Fig. 20). This hyperphosphorylation is reversible if the treatment with Aβ 1-42 is relatively short (2 to 10 min), but it becomes irreversible after 20 min (Fig. 21). The release of hyperphosphorylated tau results in decomposition of the

microtubular system (Fig. 18/C/D); if the hyperphosphorylated tau proteins form aggregates (paired helical filaments; Torreilles *et al.*, 2002), the decomposition is irreversible. Intracellular tau aggregation is also a time-dependent process, this might be the explanation of the time dependence of the cytoskeletal deorganization caused by Aβ 1-42 (Grace *et al.*, 2002; 2003).

The study of the neurotoxicity of Aβ 1-42 by the means of MTT assay (cellular redox activity) and Neutral Red assay (active membrane uptake) provided another evidence of the time dependence of irreversibility (Fig. 16). The treatment of the cells with Aβ 1-42 led to an irreversible decrease in cell viability after very short time (2 min). Although the tau hyperphosphorylation becomes irreversible only after a longer period (more than 10 min), other changes in cell viability (e.g. factors involved in generating the redox potential of the cell) are irreversible within a short period of time. (The MTT assay measures the result of very complex processes in which reduced coenzymes and ATP are formed; Liu *et al.*, 1997a; 1997b.) Rapid deterioration of the cell viability (superoxid generation, Fig. 22; lipid peroxidation, Fig. 23; increase of the intracellular calcium level, Fig. 24) and destruction of the cytoskeletal elements together induce changes in cell morphology (neurite degeneration) in the neuroblastoma culture. Similar processes might be responsible for the *in vivo* neuronal degeneration (Brandt, 2001; Hamdane *et al.*, 2003). In our experiments, 2 to 10 min contact of Aβ 1-42 aggregates (10 μM) with neuroblastoma cells caused irreversible changes.

The mechanism of amyloid neurotoxicity in part is mediated by activation and clusterization of the adhesion molecules (ex. integrins; Grace *et al.*, 2002; 2003 and Cotman *et al.*, 1998). Aβ aggregates inhibited cell attachment to the extracellular matrix (wells surface) similarly with integrin like antibody and the fibronectin analog RGD peptides (Fig. 25).

Some short Aβ-sequences are known as β-sheet breakers (BSBs; Tjernberg *et al.*, 1996; Soto *et al.*, 1998). The pentapeptide LPYFDn was designed on the basis of Soto's well-known BSB-peptide LPFFD. In order to increase the binding force to Aβ peptides, two changes in the structure were performed: 1) one phenylalanine was replaced with tyrosine: the additional phenolic OH-group increases the binding affinity; 2) the C-terminal carboxylate anion ($-COO^-$) was replaced with carboxamide ($-CONH_2$) group to increase the binding affinity according to quantum chemical calculations (AutoDock). The new peptide, LPYFDn, has a very high binding affinity to Aβ-fibrils. This peptide proved to be an excellent inhibitor of the neurotoxic events triggered by Aβ 1-42 aggregates and almost completely prevented oxidative stress (Fig. 22 and 23), the increase of the intracellular calcium level (Fig. 24) and the tau

hyperphosphorylation caused by Aβ 1-42 (Fig. 20). In the MTT assay, LPYFDn in 5-fold molar excess prevented the neurotoxic effect of Aβ 1-42 (pGn was ineffective in this test; Fig. 15). The pGn peptide was used because it does not have side chain groups and any determined conformation so it has practically no binding affinity to protein surfaces. LPYFDn partly prevented the binding of Aβ 1-42 clusters to SH-SY5Y cells and neurite degeneration, both after 5 min and/or after 12 hours co-aggregation with Aβ 1-42 (Fig. 13).

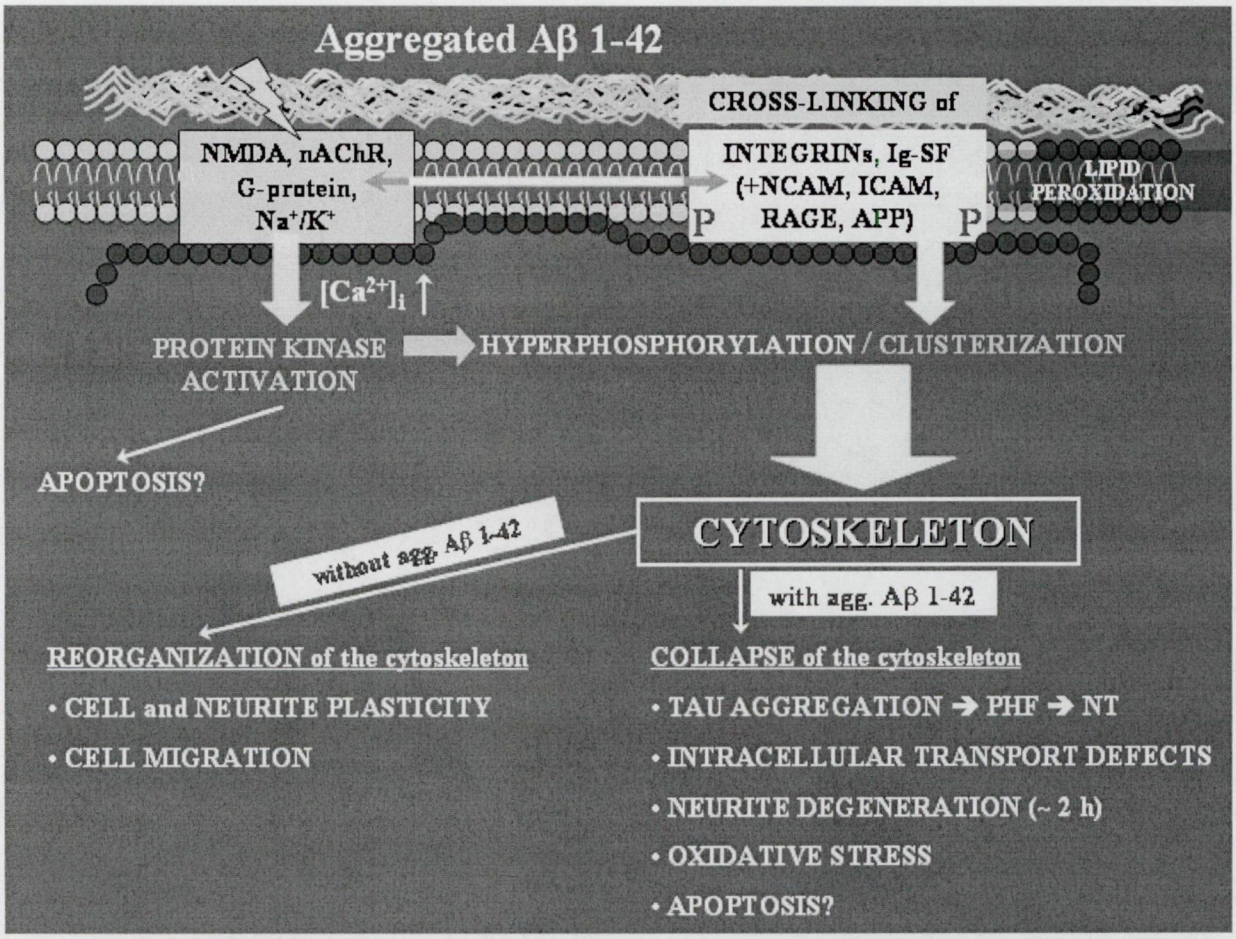


Fig. 28. *Hypothetic mechanism of the in vitro neurotoxicity of the aggregated Aβ 1-42 peptide in differentiated SH-SY5Y neuroblastoma cells. Aggregated Aβ 1-42 cause: protein kinase activation, hyperphosphorylation, clusterization of the adhesion molecules and lipid peroxidation. The protein kinase activation alone is not enough for the induction of the apoptotic cascade. Hyperphosphorylation and clusterization together cause the collapse of the cytoskeleton (intracellular transport defects, neurite degeneration). The normal functions of the cytoskeleton (cell plasticity, cell migration) are damaged.*

The mechanism of the neuroprotective effect of our pentapeptides is not perfectly clear. In a cell-free experiment, LPYFDn competes with CR for the binding sites of the Aβ surface, and after co-aggregation of Aβ 1-42 and LPYFDn for 24 h, no CR binding can be observed.

The co-aggregation of pGn with Aβ 1-42 causes practically no change in CR binding (Fig. 26). Interestingly, LPYFDn can not prevent the formation of Aβ fibrils and spherical clusters; the amount of Aβ aggregates remains unchanged after LPYFDn treatment. Electron microscopic studies also demonstrate that the co-aggregation of Aβ with LPYFDn for 1 to 3 days does not disturb fibril formation (to be published elsewhere). Molecular simulation (AutoDock) calculations reveal that LPYFDn can be tightly bound to the surface of Aβ 1-42 (Hetényi *et al.*, 2002a; Hetényi *et al.,* 2002b; Fig. 27). We presume that the pentapeptide LPYFDn (and perhaps also other short Aβ peptides) binds to the surface of Aβ aggregates, thereby preventing the interaction of Aβ 1-42 clusters with the membrane proteins of the neurons. The degree of polymerization and the fibrillar structure of Aβ 1-42 may remain unchanged, but the surface is covered with short peptide fragments, amyloid surface covering molecules (ASCOM) that prevent cell adhesion and neurotoxicity. These short protective peptides could be beneficial for the therapeutic treatment of AD (Soto *et al.,* 1998; 2000; Sigurdsson *et al.,* 2000). Further studies are necessary to explain the exact neuroprotective mechanism of short peptides.

6. Summary

Many neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, prion- and Huntington's disease are characterized by neural damage that may be caused by toxic, abnormal, aggregation-prone-proteins. The mechanism of AD specific neurodegeneration is not clearly understood yet. Since investigation of any biochemical and cell-physiological events are impossible in AD human brain, monkey, rat or mouse models are used which are more similar to human specific neurodegenerative events than *in vitro* cell culture models. However, owing to high expenses and timing, I used *in vitro* methods instead of *in vivo* ones. In addition, screening of drug candidates at the early stage of research need fast *in vitro* cell culture methodologies. My research field also involves the *in vitro* modelling of the Alzheimer's disease specific neurodegeneration and neuroprotection in cell cultures.

Different methods were used to investigate the mechanism of action of Aβ peptides on neuroblastoma cells: viability assays, fluorescence microscopy, confocal and transmission electron microscopy, immunocytochemistry.

Our results:

1. We worked out a neurotoxicity assay using fully differentiated SH-SY5Y human derived neuroblastoma cells;

2. The *in vitro* results correspond to the markers which are characteristic of AD. These markers are the following: neurite degeneration, oxidative stress, increase of the intracellular calcium level, tau hyperphosphorylation and collapse of cytoskeleton after the Aβ or NAC treatment;

3. AD can be modelled by the mutual use of full-differentiated human neuroblastoma cell culture and aggregated (diffusible or fibrillar) polypeptides such as Aβ and NAC;

4. Aggregated Aβ 1-42 induced irreversible changes in the neurite morphology. Changes in cell viability and physiology are irreversible during the first hour after the addition of Aβ 1-42 to the cells. These rapid events indicate that Aβ might induce neurodegeneration even at an early stage of the Aβ - cell contact;

5. We have shown that the novel pentapeptide, LPYFDn, an analog of Soto's LPFFD, significantly decreased the neurotoxic effect (cell viability reduction) induced by Aβ 1-42. This pentapeptide blocks the deleterious effects of the Aβ 1-42;

6. These results suggest that peptidomimetic derivatives of LPYFDn (patent 2005) can be possible new drug candidates preventing Alzheimer's disease.

7. Acknowledgements

Thanks for the help and support of the following persons: Prof. Dr. Botond Penke for the adherent education; Lívia Fülöp for the TEM experiments; Dr. Katalin Soós and Dr. Márta Zarándi for the synthesis of the peptides; Dr. Csaba Hetényi for the blind docking; Dr. Anna Juhász for her useful pieces of advice; Rita Papp and Dénes Zádori for their help in the experiments.

Supported by the following grants: OTKA O-34895, OTKA T-038236, EU-5 "LIPIDIET" (QLRT-2001-00172), NKFP 1/027/2001 and NKFP 1/040/2001. C.H. is a Békésy fellow of the Hungarian Ministry of Education.

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